RNA-binding proteins in yeast mitochondria

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von
Claudia D. Deumer
aus Gera

Gutachter: 1. Prof. Dr. Gerhard Rödel
2. Prof. Dr. Leslie A. Grivell
3. Prof. Dr. Michael Göttfert

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Abbreviations

Ace	Acetate
AMP	Adenosine monophosphate
APS	Ammoniumperoxidisulfat
ATP	Adenosine triphosphate
c	Cytosolic
COB	Cytochrome *b*
COX	Cytochrome *c* oxidase
cpm	Counts per minute
C-terminus	Carboxyl-terminus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonuleic acid
dNTP	Deoxynucleosidtriphosphate
DTT	Dithiothreitol
EDTA	Ethylendiamine-tetraacetic acid
EGFP	Enhanced green fluorescent protein
Gly	Glycerol
HA	Heme agglutinine
HRP	Horseradish-peroxidase
IM	Inner membrane
IMS	Intermembrane space
kDa	Kilodalton
min	Minutes
mRNA	Messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form of NAD^+)
N-terminus	Amino-terminus
nt	Nucleotides
OD	Optical density
OM	Outer membrane
ORF	Open reading frame
PAGE	Polyacrylamide gel-electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
poly(A)	Poly adenylate
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel-electrophoresis
TEMED	N,N,N',N'-Tetramethylethylenediamine
TM	Transmembrane
tRNA	Transfer RNA
Tris	Tris(hydroxymethyl)aminomethane
UTP	Uridine 5'-triphosphate
UTR	Untranslated region
v/v	Volume per volume
w/v	Weight per volume

Amino acids

A	Ala	alanine	M	Met	methionine
C	Cys	cysteine	N	Asn	asparagine
D	Asp	asparagine	P	Pro	proline
E	Glu	glutamate	Q	Gln	glutamine
F	Phe	phenylalanine	R	Arg	arginine
G	Gly	glycine	S	Ser	serine
H	His	histidine	T	Thr	threonine
I	Ile	isoleucine	V	Val	valine
K	Lys	lysine	W	Trp	tryptophan
L	Leu	leucine	Y	Tyr	tyrosine

Aim of the work

Mitochondrial biogenesis and function are complex biological processes that depend on the coordinate expression of (hundreds of) nuclear genes and the mitochondrial genome. For example the translation of mitochondrial mRNAs encoding subunits of the respiratory chain is activated by distinct proteins encoded in the nucleus (Fox, 1996). These mRNA-specific translational activators are associated with the inner mitochondrial membrane, recognize sites in the 5'-untranslated leaders of their target mRNAs and interact functionally with the mitochondrial ribosomal small subunit. Membrane-bound activators may be an adaptation for targeting the synthesis of mitochondrially encoded membrane proteins to the membrane. In addition, activation of translation of specific mRNAs is probably a rate-limiting step used to modulate the expression of mitochondrial genes in response to environmental conditions. Action of the various translational activators should be considered in relation to that of the bifunctional Krebs cycle enzyme NAD^+-dependent isocitrate dehydrogenase (Idhp). Idhp inhibits mitochondrial translation (de Jong *et al.*, 2000) probably mediated by binding to the 5'-UTRs of all mitochondrial mRNAs (Dekker *et al.*, 1992). In this way Idhp could coordinately control translation of all mitochondrially encoded proteins.

This work will focus on the further characterisation of Idhp and of the Krebs cycle enzymes citrate synthase 1 (Cit1p) and malate dehydrogenase 1 (Mdh1p) both of which have been identified as RNA-binding proteins. Besides analysing their effects on mitochondrial translation and their organisation in protein complexes the work focuses on the characterisation of the RNA-binding properties of recombinant Cit1p and Mdh1p. The influence of cofactors and substrates on RNA-binding will be analysed in order to reveal a possible link between the enzymatic function and the property of RNA-binding.

1 INTRODUCTION

1.1 Bifunctional proteins

Over the past few years, it has been discovered that a number of enzymes has not only a metabolic role in the cell but is also able to bind mRNAs. In that way they are involved in either translational regulation or in control of mRNA stability. These enzymes represent a growing family of proteins with dual functions. To give an impression about the diversity of the enzymatic function and the key regulatory role in a biochemical pathway, a short review on aconitase and its importance in iron homeostasis is given.

1.1.1 Aconitase

The most extensively studied member of bifunctional enzymes is the cytosolic (c) aconitase (summarised in Table 1). Its RNA-binding activity depends on the iron concentration in the cell. Therefore it is also called iron-responsive element-binding protein (IRE-BP). The IRE-BP binds to specific stem-loop RNA structures known as iron-responsive elements (IREs). IREs are present in a variety of cellular mRNAs encoding proteins involved in iron uptake (transferrin receptor (TfR)), storage (ferritin) and utilization (erythroid 5-aminolevulinate synthase (eALAS)) (Eisenstein *et al.*, 1993; Hentze and Kuhn, 1996). Expression of these genes is regulated by interaction with the IRE-BP via translational regulation (for ferritin and eALAS mRNAs) or by controlling mRNA stability (for TfR mRNA), depending on the location of the IRE (Eisenstein *et al.*, 1993; Hentze and Kuhn, 1996; Theil, 1998). IREs involved in translational regulation are located in the 5'-UTR of the mRNAs, while IREs involved in mRNA stability are located in the 3'-UTR of the mRNAs. The function of IRE-BP is determined by the presence or absence of an Fe-S cluster: it shows aconitase activity when the Fe-S cluster is present, and acts as an RNA-binding protein when the protein lacks this cluster (Kaptain *et al.*, 1991; Kennedy *et al.*, 1992; Haile *et al.*, 1992; Eisenstein *et al.*, 1993). Aconitase activity and IRE-binding activity are mutually exclusive, and interconversion between the two activities is determined by the intracellular Fe concentration. Fe-S cluster assembly occurs under conditions of excess iron, converting IRE-BP to c-aconitase and stimulating synthesis of ferritin and eALAS synthesis, while TfR synthesis is repressed. Iron depletion promotes cluster disassembly, hence conversion of c-aconitase to IRE-BP, and repression of ferritin and eALAS synthesis, while stimulating TfR expression.

Subsequently, another member of the iron regulatory protein family was identified. In the following this protein is referred to iron regulatory protein 2 (IRP2), while IRE-BP is the iron regulatory protein 1 (IRP1). IRP1 and IRP2 have a high sequence identity except for a 73-amino acid insertion unique to IRP2. In contrast to IRP1, IRP2 has only IRE-binding activity and is regulated by the degradation of the protein when cells are iron-repleted (Guo *et al.*, 1995; Iwai *et al.*, 1995, 1998; Eisenstein *et al.*, 1997).

The control of IRE-BP activity is also subject to regulation by other factors. Both IRP1 and IRP2 are phosphorylated *in vivo*, indicating that their function may be integrated into more general metabolic signals (Eisenstein *et al.*, 1993; Schalinske and Eisenstein, 1996). Exposure of cells to nitric oxide (NO) or hydrogen peroxide (H_2O_2) enhances IRE-binding activity and inhibits c-aconitase activity in animal cells (Hentze and Kuhn, 1996). Both NO and H_2O_2 cause Fe-S cluster disruption (Kennedy *et al.*, 1997; Brazzolotto *et al.*, 1999). The consequence of these regulatory processes would be to modulate the expression of genes regulated by IRP1 and aconitase activity, similar to the effect of iron on IRP1.

Functional IREs are also identified in the 5'-UTRs of the mRNAs encoding the Krebs cycle enzymes aconitase (porcine) (Dandekar *et al.*, 1991) and the iron-sulfur protein subunit of succinate dehydrogenase (Sdhp) in *Drosophila melanogaster* (Gray *et al.*, 1996). Translational repression of these genes is mediated by IRP1, thereby identifying a regulatory link between the control of iron homeostasis by the IRE/IRP system and the mitochondrial Krebs cycle (Dandekar *et al.*, 1991; Gray *et al.*, 1996). IRP-mediated changes in mitochondrial aconitase (and Sdhp) abundance may represent a means to modulate the availability of citrate in cellular iron homeostasis. Citrate can bind iron as well as promote iron uptake and release from mammalian cells. Iron citrate is a major component of the non-transferrin-bound iron pool present in the plasma in some forms of iron overload (Grootveld *et al.*, 1989), suggesting the possibility that it may be released by some tissues such as the liver. Furthermore, citrate influences the binding and/or release of iron to and from ferritin and transferrin. Given the known function of IRP1 and IRP2 in modulating iron uptake and storage, it is reasonable to assume that mediation of mitochondrial aconitase expression by IRP represents a directed effort to modulate the role of citrate in cellular trafficking.

RNA-binding activity has also been attributed to other "regular" enzymes, including thymidilate synthase, catalase, glyceraldehyde-3-phosphate dehydrogenase and glutamate dehydrogenase (Hentze, 1994). None of these proteins contain recognizable RNA-recognition motifs, all of them do, however, contain a (di-)nucleotide binding domain (Rossmann fold, Rossmann, 1975). RNA-binding of some of these enzymes, e.g. glyceraldehyde-3-phosphate

dehydrogenase, glutamate dehydrogenase, catalase and thymidylate synthase, could be competed by NAD(H), NADP(H) or ATP, suggesting that the Rossmann fold might serve as an RNA-binding domain and indicating that the enzymatic function and RNA-binding are mutually exclusive (Chu *et al.*, 1993; Nagy and Rigby, 1995; Clerch *et al.*, 1996; Bringaud *et al.*, 1997). These observations led to the hypothesis that the RNA-binding domains present in these enzymes evolved from a (di-)nucleotide binding site, or *vice versa* (Hentze, 1994).

Table 1: Properties of aconitase

RNA-binding enzyme	Enzymatic function	Target RNA	Putative RNA-binding function	Modulation of RNA-binding	Reference
Aconitase/IRP1	Conversion of citrate into isocitrate	IRE in: • 3' TfR mRNA • 5' Ft mRNA • 5' eALAS mRNA • 5' mt aconitase mRNA • 5' SDH mRNA	Protection of mRNA Translational regulation	Iron concentration NO, H_2O_2 concentration	Hentze and Kuhn, 1996

1.2 Mitochondria

Mitochondria are near ubiquitous organelles of eukaryotic cells. Their assembly and function result from a collaboration between gene products encoded by mitochondrial and nuclear genomes. Most of the proteins which reside in the mitochondrion are nuclear gene products accounting for about 90 % of the protein mass of the mitochondrion. These proteins are translated in the cytoplasm and subsequently posttranslationally transported into the mitochondrion. They play key roles in mitochondrial transcription and translation, mitochondrial lipid and heme synthesis, substrate oxidation by the Krebs cycle and mitochondrial electron transport and oxidative phosphorylation. In short, these proteins play roles in all aspects of mitochondrial function, and they are distributed in all four mitochondrial compartments (inner and outer membrane, matrix and intermembrane space). In contrast, the mitochondrial genome specifies only a few proteins, which reside mainly in the inner mitochondrial membrane. But the nuclear and the mitochondrial genomes do not only interact in the described way. The mitochondrial genome can also give a feedback to the

nucleus and affect the expression of nuclear genes for mitochondrial (and other) proteins (Parikh *et al.*, 1987; Farrell *et al.*, 1990). This communication pathway by which the mitochondrion "talks to" the nucleus is not well understood but is likely to be fundamentally different from that of the communication pathway from the nucleus to the mitochondrion. Probably it involves metabolic signals and one or more signal transduction pathways that function across the inner mitochondrial membrane.

1.2.1 Regulation of mitochondrial function

Several factors such as oxygen level, carbon source and metal ions are known to alter mitochondrial protein levels and/or function. This regulation is important for adjusting the energy producing capacity of a cell and especially developed in the regulation of respiratory activity of yeast mitochondria. Respiration undergoes dramatic changes in response to the environmental factors oxygen and glucose. Under anaerobic conditions or when grown on glucose as carbon source, synthesis of the respiratory complexes and of the ATPase is repressed. As a result mitochondria are almost totally devoid of the ability to oxidize nonfermentable substrates and to generate ATP by oxidative phosphorylation (Mahler *et al.*, 1975). When yeast cells are grown on a carbon source that requires mitochondrial function the steady-state levels of most mitochondrial mRNAs increase about 5-fold compared with growth on glucose medium (Müller and Getz, 1986). In contrast to the steady-state level of RNAs, the apparent rates of synthesis of the major mitochondrial gene products (measured by *in vivo* labelling in the presence of cycloheximide) vary over a wider range. For example, Cox1p, Cox2p, Cox3p, and cytochrome *b* were at least 19 times more slowly labelled in anaerobically grown than in aerobically grown cells (Woodrow and Schatz, 1979). Taken together, these findings suggest that modulation of mitochondrial gene expression in response to oxygen occurs at least partially at the translational level. Similarly, the release of glucose repression greatly stimulates translation of Cox3p (Falcone *et al.*, 1983), but has little effect on its mRNA level (Zennaro *et al.*, 1985). This apparent translational modulation is generally paralleled by the expression of nuclear genes coding components of the mitochondrial translation system. However, these genes are not under tight coordinate regulation and are probably controlled by several different mechanisms.

As known from the IRE-BP (see chapter 1.1.1) some of mitochondrial functions can be regulated by metals like iron. Iron modulates the expression of the Krebs cycle enzyme aconitase via a translational mechanism involving iron regulatory proteins and interestingly, as shown recently, iron also affects three other Krebs cycle enzymes, namely citrate synthase,

isocitrate dehydrogenase and succinate dehydrogenase (Oexle *et al.*, 1999). Whereas the *SDH* mRNA of *Drosophila melanogaster* contains a functional IRE, such an element has not been identified so far in the untranslated regions of *CIT* or *IDH* mRNA. Therefore the enzymatic activities of these proteins apparently are not regulated by the IRP/IRE system. Since the Krebs cycle provides NADH for oxidative phosphorylation to generate the proton gradient for ATP formation, influences on Krebs cycle enzymes can cause broad changes. Indeed iron supplementation leads to increased formation of reducing equivalents (NADH) by the Krebs cycle and thus results in increased mitochondrial oxygen consumption and ATP formation via oxidative phosphorylation (Oexle *et al.*, 1999).
These results point to an interaction of both oxygen supply and cellular energy metabolism with iron homeostasis and give an idea of the complex regulation by environmental factors.

1.2.1.1 Organisation of the respiratory chain

The mitochondrial electron transport chain is composed of four complexes embedded in the mitochondrial inner membrane: complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinol cytochrome *c* reductase) and complex IV (cytochrome *c* oxidase). In contrast to most eukaryotes, the yeast S*accharomyces cerevisiae* (*S. cerevisiae*) does not possess a complex I, but rather contains two NADH dehydrogenases associated with the inner membrane (de Vries and Marres, 1987; Luttik *et al.*, 1998). The respiratory chain complexes are electronically linked by ubiquinone and cytochrome *c*. They act in sequence to accept reducing equivalents from NADH or $FADH_2$ and transfer them through a series of oxidation-reduction reactions to O_2, resulting in the formation of water and the generation of proton and ion gradients across the inner mitochondrial membrane. These latter gradients are used to drive the synthesis of ATP by another multimeric protein, complex V (ATP synthase). Investigations concerning the mobility of ubiquinone and cytochrome *c* showed, that these electron carriers do not diffuse freely through or along the membrane (Boumans *et al.*, 1998). From these findings it was concluded that, at least in yeast mitochondria, the respiratory chain is physically connected and may act as a functional unit. Cruciat *et al.* (2000) showed that complex III is associated with complex IV. Physical association of the mitochondrial respiratory chain complexes may serve to enhance the flow of electrons between these complexes and to reduce the dependency on random diffusion of the electron carriers ubiquinone and cytochrome *c*. Indeed formation of a supracomplex between complexes III and IV in *Paracoccus denitrificans* has been demonstrated to significantly enhance electron transfer between these complexes (Berry and

Trumpower, 1985). Furthermore Schägger and Pfeiffer (2000) showed that cytochrome *c* oxidase of *S. cerevisiae* is bound to cytochrome *c* reductase which exists in three forms: the free dimer, and two supercomplexes comprising an additional one or two complex IV monomers. The distribution between these forms depends on the carbon source. In mammalian mitochondria, complex I and III can also form a supercomplex containing up to four copies of complex IV. These data guided the authors to develop a model for a network of respiratory chain complexes called "respirasome" (Schägger and Pfeiffer, 2000). Such multienzyme complexes mediate substrate channelling which allows transfer of an intermediate to a specific enzyme without competition by other enzymes. A major advantage of substrate channelling is the use of a localised substrate molecule (potential candidates are ubiquinone and cytochrome *c*), which makes a reaction independent of a substrate pool. The absence of a pool function for cytochrome *c* in yeast indicates tight substrate channelling which consequently leads to catalytic enhancement, sequestration of reactive intermediates and rapid intramolecular group transfer reaction (Fersht, 1999; Schägger and Pfeiffer, 2000).

1.2.1.2 Organisation of the Krebs cycle

The Krebs cycle plays a central role in anabolism by providing components for biosynthetic pathways and in catabolism by oxidative decarboxylation of metabolites. During the decarboxylation step the coenzyme NAD^+ is reduced. These reducing equivalents are oxidised via the respiratory chain. Thus a coenzyme and its regeneration links two different pathways (Fig. 1). For the respiratory chain, an interaction of the enzymes with each other which allows the formation of highly organised enzyme complexes was outlined above (1.2.1.1). The kinetic advantage of such "organised enzymes" has also been demonstrated for the Krebs cycle (Robinson *et al.*, 1987) while direct evidence for physical contacts between the Krebs cycle enzymes was only obtained for several enzymes: binding of malate dehydrogenase to complex I in beef heart mitochondria (Ovadi *et al.*, 1994), channelling of oxaloacetate by malate dehydrogenase and citrate synthase (Lindbladh *et al.*, 1994) and channelling of citrate by citrate synthase and aconitase (Velot and Srere, 2000). The observation that Citp behaves as an immobilized enzyme suggests its participation in a supramolecular complex (Haggie and Brindle, 1999). These data led to the development of the "metabolon" concept describing a possible Krebs cycle complex. Recently Grandier-Vazeille *et al.* (2001) presented results supporting this idea of a metabolon and indicating an interaction of the Krebs cycle with the respiratory chain. The authors detected in yeast a supramolecular complex containing two NADH dehydrogenases and four enzymes of the Krebs cycle, namely Mdh1p, Cit1p, Sdh1p

and Fum1p (fumarate hydratase). Fum1p catalyses the metabolic step preceding Mdh1p suggesting a channelling between these enzymes, while Cit1p catalyses the metabolic step following Mdh1p indicating a channelling between these two enzymes, too. The assumption of Cit1p as a relatively immobilized protein (Haggie and Brindle, 1999) which is associated with a supramolecular complex was confirmed by its participation in the described NADH dehydrogenase complex. Sdh1p catalyses the step preceding Fum1p also indicating a channelling. The association of these proteins with the proteins of complex I may indicate NADH-channeling and besides the obvious functional connections may hint to physical contacts of Krebs cycle components and respiratory chain enzymes.

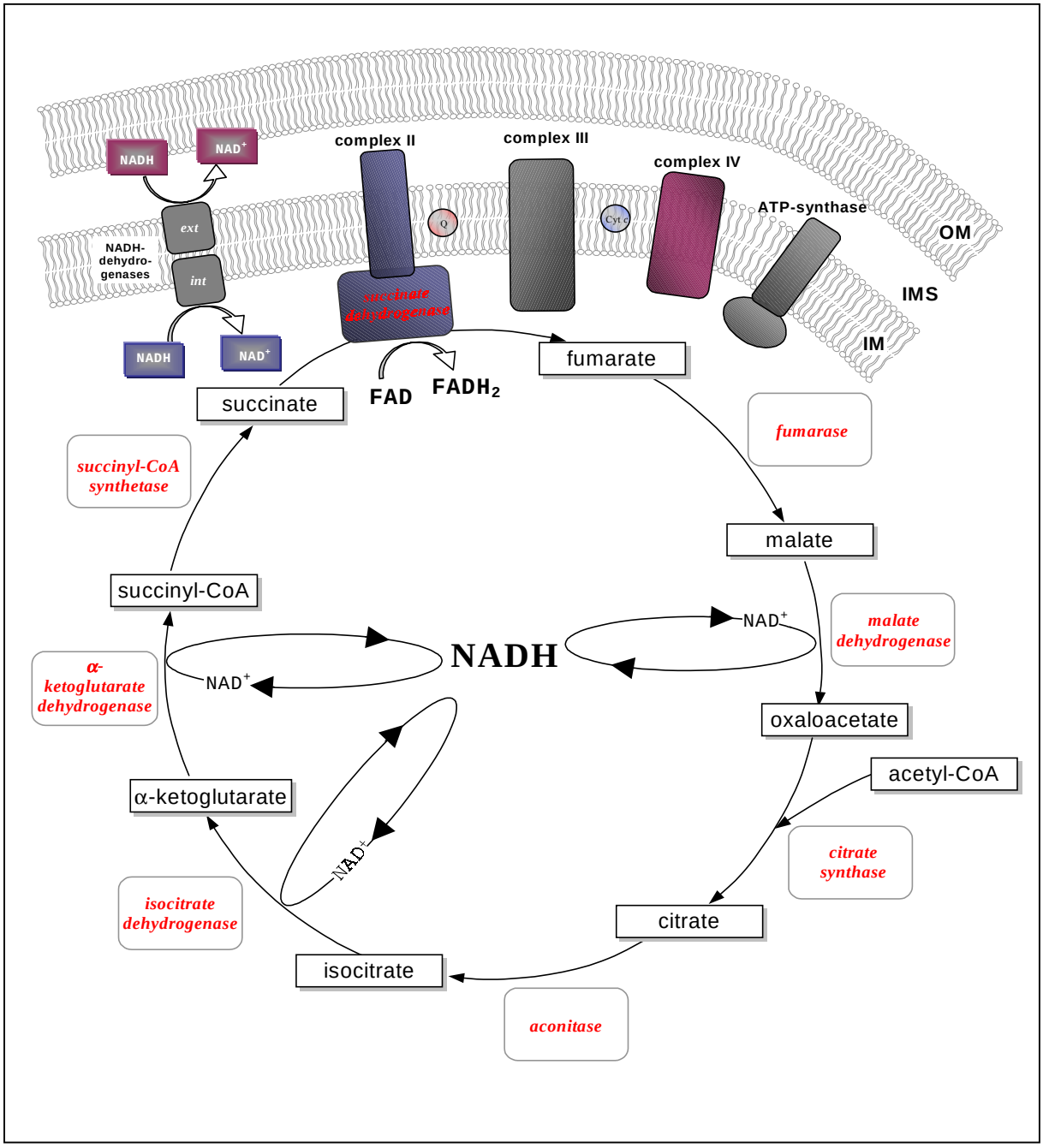


Fig. 1: Functional connection between respiratory chain and Krebs cycle
The respiratory chain complexes are located in the mitochondrial inner membrane (IM), and the Krebs cycle enzymes in the mitochondrial matrix. Both pathways are linked by the coenzyme NAD^+ and its regeneration. During the Krebs cycle NAD^+ is reduced. The reducing equivalents NADH drive the respiratory chain where they will be oxidised.

1.2.2 Regulation of mitochondrial translation

Gene expression in yeast mitochondria is mainly regulated at the translational level (Pel and Grivell, 1994). By far most of the proteins known to be involved in expression of mitochondrial genes are required for translation while only a few have been shown to be necessary for transcription.

Initiation of translation includes the sequential recruitment of a small and a large ribosomal subunit to the mRNA, to form a translation-competent ribosome at the appropriate initiation codon. In case of cytoplasmic mRNAs, it requires the participation of many eukaryotic initiation factors as well as the cap structure at the 5'-end and a poly(A) tail at the 3'-end of the mRNA. The 5'-cap, which is present on nearly all eukaryotic mRNAs, facilitates the initiation of translation while the poly(A) tail is thought to stabilise the mRNA by protecting it against degradation. The poly(A) tail gradually shortens in time, indicating that it may fix the half life of the mRNA. These mechanisms are not used in yeast mitochondrial mRNAs as they are uncapped (Christianson and Rabinowitz, 1983) and lack a poly(A) tail (Moorman *et al.*, 1978). Although mitochondrial and eubacterial translation systems show many similarities, they also exhibit differences in the mechanism of translational initiation. In eubacteria, bin-ding and positioning of mRNA on the small ribosomal subunit depends on base pairing bet-ween a sequence in the 16S rRNA and a complementary sequence in the 5'-untranslated region of the mRNA, known as the Shine-Dalgarno sequence (Gold, 1988). This kind of interaction seems unlikely in yeast mitochondria, since there are no obvious Shine-Dalgarno sequences present in mitochondrial mRNAs. Moreover scanning for initiation codons by the ribosome, which is proposed for the cytosolic counterparts in eukaryotes (Kozak, 1983), seems unlikely to occur since the 5'-UTR of mitochondrial mRNAs often contain AUG initiation codons, short upstream open reading frames (uORFs) and stable secondary structures formed by short G+C rich clusters (Pel and Grivell, 1994). Stable secondary structures in leader sequences hinder the scanning by the 40S ribosomal subunit in both mammalian cells and yeast (Herrick *et al.*, 1990; Kozak, 1991a; Vega Laso *et al.*, 1993; Beelman and Parker, 1994; Sagliocco *et al.*, 1994). uORFs are seldom found in cytosolic

transcripts of yeast and higher eukaryotes (Kozak, 1991b) where they can have a negative effect on the efficiency of translational initiation at the authentic start codon (Hinnebusch *et al.*, 1988). In some cases uORFs might exhibit regulatory functions (Abastado *et al.*, 1991). Therefore mitochondrial ribosomes can probably not reach the start codon by scanning of the untranslated leader RNAs, but rather bind at an internal site of the mRNA-leaders. The mechanism of this process is still unclear. Internal translational initiation has been shown for Picorna-viral RNA in eukaryotic cells using conserved structural domains in the 5'-UTR of the viral RNA genome. The Picornavirus RNA does not have a 5'-cap but has a long untranslated region at the 5'-end. This region forms stem-loop structures which interact with the 40S ribosomal subunit and initiate the cap-independent translation at the internal ribosome entry site (IRES) (Agol, 1991; Belsham and Sonenberg, 1996; Stewart and Semler, 1998; McCarthy, 2000). Izquierdo and Cuezva (2000) have shown for mammalian cells that the RNA of the nuclearly encoded β-subunit of the mitochondrial ATP-synthase is regulated by an enhancer sequence of 150 bp located in the 3'-untranslated region. By means of transcription of a reporter gene it was shown that this enhancer sequence initiates transcriptional start independent of its localisation at the 5'- or the 3'-end. If the enhancer sequence is located at the 3'-end, an interaction between the 5'- and the 3'-end is required during translation initiation (Izquierdo and Cuezva, 2000). In addition the 5'-leader sequences of *YAP1*- and *p150*-mRNA of *S. cerevisiae* also contain functional IRES elements (Zhou *et al.*, 2001).

A ribosome binding site with a fixed position relative to the start site of translation, like those of prokaryotes, seems not to be present in mitochondrial mRNAs. While sites in the 5'-leaders complementary to the 3'-end of the 15S rRNA have been noted (Li *et al.*, 1982), their positions vary between -8 and -107 relative to the initiator AUG codon. Furthermore, in the case of cytochrome *b* (*COB*) deletion of the sequence motif complementary to the 3'-end of the 15S rRNA did not cause any changes in *COB* expression (Mittelmeier and Dieckmann, 1995). Green-Willms *et al.* (1998) have shown that defined mutations of the 5'-mRNA leader of *COX2* and *COX3* can be suppressed by alterations of mRNA-specific activators and by mutations of mitochondrial ribosomal proteins. These components of the small ribosomal subunit, namely Mrp21p and Mrp51p, cause a suppression which is specific for certain *COX2* and *COX3* alleles. Therefore the authors assume that yeast mitochondrial ribosomes play an active role in the recognition of the translational initiation signal. Since the suppression concerns both alleles of *COX2* and *COX3*, it is not gene specific and may indicate that ribosomes re-cognise regions in the 5'-leaders of different mRNAs. Possibly there is a

common sequence motif which is contained in all mitochondrial mRNA leader sequences. The octanucleotide sequence UAUAAAUA found in the leader regions of yeast mitochondrial mRNAs could represent this motif (Dunstan *et al.*, 1997). This sequence is complementary to several regions of the mitochondrial 15S rRNA and might be involved in the mRNA-rRNA basepairing. Proteins like Mrp21p and Mrp51p could be involved in such an mRNA-rRNA interaction (Green-Willms *et al.*, 1998). Since the octanucleotide sequence is not at a fixed position relative to the translational start and the complementary sequence is not found at the 3'-end of the rRNA, this interaction is not comparable to the Shine-Dalgarno mechanism (Shine and Dalgarno, 1974; Ringquist *et al.*, 1992). Alternatively mitochondrial ribosomes could directly interact with mRNAs under participation of Mrp21p and Mrp51p (Green-Willms *et al.*, 1998). The unusual feature of the yeast mitochondrial translation system that nuclearly encoded mRNA-specific translational activators exist could also point to an unknown mechanism of translation initiation.

1.2.2.1 Specific translational factors

The mitochondrial translation of at least five of the eight major mRNAs requires the action of one or more nuclear encoded gene-specific activator proteins (Table 2). These activator proteins are thought to recognise sites in the 5'-UTR of the mRNA (Jackson *et al.*, 1990) and to interact with the mitoribosomal small subunit (Haffter *et al.*, 1991). All translational activators are associated with the inner mitochondrial membrane (McMullin and Fox, 1993). Through these interactions the insertion of translation products into the appropriate respiratory complexes might be facilitated.

Table 2: Factors involved in translation of specific yeast mitochondrial mRNAs

mRNA	Factor	Localisation	Ribosomal interaction	References
COX1	Mss51p	associated with inner membrane	n.d.	Siep *et al.*, 2000 Decoster *et al.*, 1990
	Pet309p	inner membrane		Manthey and McEwen, 1995
COX2	Pet111p	inner membrane	n.d.	Poutre and Fox, 1987
COX3	Pet54p	associated with inner membrane	n.d.	Costanzo *et al.*, 1986
	Pet122p	inner membrane	yes	Kloeckener-Gruissem *et al.*, 1988
	Pet494p	inner membrane	n.d.	Müller *et al.*, 1984

COB	Cbs1p	inner membrane	n.d.	Rödel, 1986a
	Cbs2p	associated with inner membrane	yes	Rödel *et al.*, 1986b
	Cbp6p	n.d.	n.d.	Dieckmann and Tzagoloff, 1985
ATP9	Aep1p	n.d.	n.d.	Payne *et al.*, 1993
	Aep2p	n.d.	n.d.	Finnegan *et al.*, 1991

Three nuclear genes, *PET494*, *PET54* and *PET122*, are specifically required for *COX3* mRNA translation. Mutations in each of these genes abolish translation of a chimeric mRNA bearing the *COX3* 5'-UTR and the coding sequence of *COB*, showing that all three products act within the *COX3* 5'-UTR (Costanzo and Fox, 1988). Mutation of the translation initiation codon AUG to AUA had no effect on the levels of Cox3p, showing that the site of interaction is specified by other sequence or structural features (Folley and Fox, 1991). Deletion of the entire 5'-UTR completely prevented translation. Pet494p, Pet54p and Pet122p probably work together to carry out a similiar function. Using a *GAL4*-based two-hybrid system physical interactions have been detected between Pet54p and Pet122p, and between Pet54p and Pet494p (Brown *et al.*, 1994). Genetic analysis of *pet54* mutants also revealed functional interactions between Pet54p and Pet122p. Localization studies showed that Pet122p and Pet494p are integral inner membrane proteins, while half of the Pet54p is associated with the inner membrane and the other half is in soluble form (McMullin and Fox, 1993). Besides making contact with *COX3* mRNA and the mitochondrial membrane, the Pet494p/54p/122p complex is likely to interact with the mitochondrial ribosome. Mutations of *PET123*, *MRP1* and *MRP17* were identified to partially compensate for the loss of the C-terminus of Pet122p (McMullin *et al.*, 1990; Hafter *et al.*, 1990, 1991; Hafter and Fox, 1992). All three genes encode proteins of the small mitoribosomal subunit. These findings indicate that Pet122p promotes translation initiation of *COX3* mRNA through an interaction with the small subunit of the mitoribosome. Taken together, all data point to a complex formed by the activators that mediates the interaction of the *COX3* mRNA with the mitoribosome at the surface of the inner membrane.

A similar picture arises for Cbs1p and Cbs2p, two proteins required for translation of *COB* mRNA (Rödel, 1986a). The respiratory deficiency due to mutations in the genes *CBS1* and *CBS2* can be suppressed by mitochondrial mutations replacing the *COB* leader by the untranslated leader of the *ATP9* gene (Rödel *et al.*, 1986b). Analysis of deletions in the 5'-

UTR of *COB* mRNA mapped the site of action of the translational activators either between –170 and –104 or between –60 and the AUG at +1 (Mittelmeier and Dieckmann, 1995). Cbs1p behaves as an integral inner membrane protein, while Cbs2p behaves as a soluble protein which is associated with the inner membrane via ionic interactions (Michaelis *et al.*, 1991). A two-dimensional separation of small mitoribosomal subunit proteins indicated an association of Cbs2p with mitochondrial ribosomes (Michaelis *et al.*, 1991) which was confirmed by coimmunoprecipitation of Cbs2p with the mitoribosomal protein Nam9p (Tzschoppe, 2001).

Translation of *COX2* mRNA is dependent on Pet111p (Poutre and Fox, 1987). Similar to other translational activators, Pet111p acts via the 5'-UTR of the mRNA. Studies with chimeric mRNA bearing the *COX3* 5'-UTR and the coding sequence of *COX2* showed that Pet111p acts specifically at a site in the 54 nucleotides long *COX2* 5'-leader (Mulero and Fox, 1993a). A translational defect resulting from a point mutation generated in the 5'-UTR of *COX2* mRNA could be suppressed by a missense mutation in the *PET111* gene (Mulero and Fox, 1993b). Further mutational analysis revealed functional elements as well as a possible stem loop structure at –20 to –35 in the 5'-untranslated region of *COX2* mRNA (Dunstan *et al.*, 1997). Like Pet122p and Pet494p, Pet111p has been detected in mitochondria as a tightly bound inner membrane protein (Strick and Fox, 1987). So far no functional link between Pet111p and the mitochondrial ribosome has been demonstrated.

1.3 Isocitrate dehydrogenase

An additional protein that binds specifically and with high affinity to the 5'-untranslated leaders of all major yeast mitochondrial mRNAs has been identified by mobility shift assays with mitochondrial extracts (Papadopoulou *et al.*, 1990; Dekker *et al.*, 1992). On SDS-PAGE this protein behaved as a doublet with an approximate size of 40 kDa (p40). Peptide sequence analysis identified p40 as the Krebs cycle enzyme (NAD^+)-dependent isocitrate dehydrogenase (Idhp) (Elzinga *et al.*, 1993). Idhp is present in all eukaryotic cells and catalyses a rate-limiting step in the Krebs cycle, namely the conversion of isocitrate to α-ketoglutarate coupled to the production of NADH. It is an allosterically regulated enzyme that in *S. cerevisiae* functions as an octamer composed of two nonidentical subunits, termed Idh1p (Mr 40 kDa) and Idh2p (Mr 39 kDa) (Keys and McAlister-Henn, 1990; Cupp and McAlister-Henn, 1991, 1992). Both enzyme and RNA-binding activities are specifically lost in cells containing disruptions in either *IDH1* or *IDH2*, the nuclear genes encoding the two

subunits of the enzyme (Elzinga *et al.*, 1993), thus demonstrating that both activities are dependent on the simultaneous presence of both subunits. Furthermore mutants that result in the loss of one or both Idhp subunits are unable to grow on the non-fermentable carbon source acetate, a phenotype shared with other Krebs cycle mutants, and grow very poorly on the non-fermentable carbon source glycerol (Cupp and McAlister-Henn, 1992). The contribution of each subunit to regulation and catalysis was determined by mutation of a conserved serine residue at the proposed active site of each subunit (Cupp and McAlister-Henn, 1993). An S98A replacement in Idh2p resulted in a 60-fold decrease in V_{max}, but had no effect on cooperativity with respect to isocitrate or AMP, whereas an S92A replacement in Idh1p resulted in a 6-fold decrease in V_{max}, with complete loss of activation by AMP and a 2-fold decrease in cooperativity. Therefore a primary role for Idh2p in catalysis and for Idh1p in regulating the catalytic properties of Idh2p was suggested. Mutant proteins with affected enzymatic activity (Idh1p^{S92A}, Idh2p^{S98A}) have RNA-binding activity (Elzinga *et al.*, 1993), while the mutant protein that affects RNA-binding activity (Idh1p$^{K182L/Y184N}$) has normal enzymatic activity (Siep, 2001).

Idhp is just one of a rapidly growing family of bifunctional RNA-binding proteins. Although it is an NAD^+-binding protein containing a (di)nucleotide binding domain, RNA-binding could not be inhibited by (di-)nucleotides, suggesting that a region distinct from the Rossmann fold is involved in binding RNA (Siep, 2001). As the substrate isocitrate also did not inhibit RNA-binding the active site of the enzyme is not involved in RNA-binding (Siep, 2001). RNA-binding could, however, be involved in regulation of the enzyme: addition of *COX2* leader RNA inhibits the enzymatic activity of Idhp (Anderson *et al.*, 2000) indicating a regulatory mechanism to switch between the RNA-binding and the enzymatic function. This mRNA is a potent steric inhibitor of Idhp enzymatic activity and this inhibition is relieved by the presence of the allosteric activator AMP. Therefore the mitochondrial AMP level could also be responsible for the switch between the two Idhp functions (Anderson *et al.*, 2000).

The binding region of Idhp is still unknown, but based on sequence comparison between *K. lactis* Idh1p and *S. cerevisiae* Idh1p a region potentially involved in RNA-binding (amino acids 170 to 190) was identified (Siep, 2001). From a 3D-structure based on *E. coli* $NADP^+$-Idhp (Thorsness and Koshland, 1987), a cleft region dividing the subunit in a large and a small domain was identified. Three aromatic residues of the potential RNA-binding region (K182, K183, Y184) were notably sticking in the cleft. A mutant protein in which lysine 182 is changed to leucine and tyrosine 184 to asparagine (Idh1p$^{K182L/Y184N}$) showed strongly reduced RNA-binding capacity and normal enzymatic activity (Siep, 2001). Since

Idhp of *S. pombe* is able to bind RNA with an affinity comparable to *S. cerevisiae* Idhp (Elzinga, 2000) although Idh1p contains a leucine at position 182 and an asparagine at position 184, it is unlikely that the mutated residues are directly involved in RNA-binding and suggest that other determinants are likely to play a role.

Concerning the protein-RNA interactions it is also important to consider the sequence and the 3D-structure of the respective RNA. RNA is a dynamic molecule with the ability to fold back through base pairing of complementary stretches to form secondary structures such as hairpins, bulges and internal loops. Interestingly, the possible stem loop at –20 to –35 of the *COX2* mRNA which is bound by Idhp (Dekker *et al.*, 1992) contains the sequence UCUAA, a motif that is conserved in a number of mitochondrial yeast mRNAs 18 to 37 bases upstream of the initiation codon. Mutational analysis reveals this region as a good candidate site for Pet111p action (Mulero and Fox, 1993b). Therefore, binding of Idhp and Pet111p could be mutually exclusive so that translation could be prevented until Idhp is replaced by Pet111p and/or other components of an initiation complex causing the mRNAs to be tethered to the inner membrane.

The idea that Idhp is acting as a translational suppressor is supported by protein labelling experiments with strains lacking one of the Idhp subunits (de Jong *et al.*, 2000). The absence of Idhp resulted in an increase of translational activity in isolated mitochondria. However, these newly produced proteins are degraded more rapidly resulting in a lowered steady-state level of respiratory chain proteins. The increased turnover observed in Idhp-deficient cells is consistent with the observation that mRNAs for Cox2p and Cox3p are prevented from membrane-tethering/-docking by a replacement of their 5'-UTR through the *VAR1* 5'-UTR (Sanchirico *et al.*, 1998). The synthesis of Var1p, a protein of the mitoribosomal small subunit, should not be dependent on cotranslational membrane insertion. Indeed, the incorporation of Cox2p and Cox3p which were efficiently translated from the chimeric mRNAs was found to be severely defective (Sanchirico *et al.*, 1998). In addition, the increased production of some membrane localised respiratory chain subunits and their subsequent increased degradation presumably triggers the induction of suppressor mutations. Gadde and McCammon (1997) made the interesting observation that yeast strains with Idh2p nonsense or null mutants initially grow very poorly on glycerol but accumulate extragenic mutations, termed glycerol suppressors, that enhance growth on this nonfermentable carbon source. Missense, nonsense and null mutations at the *CIT1* locus encoding mitochondrial citrate synthase were the most common suppressors identified. In addition to *CIT1*, 3 other Krebs cycle enzyme genes are capable of suppressing *idh2* mutations (Przybyla-Zawislak *et*

al., 1999). Genetic evidence shows that glycerol suppressor mutations only occur in Idhp deficient mutants, indicating that Idhp plays a unique role in *S. cerevisiae* growing on glycerol. Mutations in other Krebs cycle enzymes do not give rise to suppressor mutations which indicates that it is not a defective Krebs cycle which leads to these mutations (Przybyla-Zawislak *et al.*, 1999). Thus, it is more likely that instead of a defective enzymatic function a defective RNA-binding function of Idhp gives rise to suppressor mutations. Possibly these suppressor mutations can compensate for the absence of the Idhp RNA-binding function. Therefore other Krebs cycle enzymes should be examined for RNA-binding affinity.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments

Bio-Imaging Analyzer BAS-1800 II	FUJIFILM
Chart Recorder 1327	BIO-RAD
Dounce homogeniser	BRAUN
Econo Gradient Monitor	BIO-RAD
Econo Pump	BIO-RAD
Econo UV Monitor	BIO-RAD
Eraser	RAYTEST
Fluor-S™ MultiImager	BIO-RAD
Fraction Collector 2110	BIO-RAD
LI-COR DNA sequencer (model 4000/4200;)	MWG-BIOTECH
Rainbow Scanner	SLT LABINSTRUMENTS
Sonoplus	BANDELIN ELECTRONIC

2.1.2 Materials

Acetyl-CoA	ICN
Acrylamide/Bisacrylamide	ROTH, ICN
AMP	ICN
ATP	ICN
Blue Sepharose CL-6B	AMERSHAM PHARMACIA BIOTECH
calf liver RNA Type IV	SIGMA
Centricon® YM-10	MILLIPORE
Coomassie® Brilliant blue G250	MERCK
Collodion Bags	SARTORIUS
Citric Acid	ICN
Dithiothreitol	SIGMA
dNTP's	INVITROGEN
DTNB	ICN
ECL^{plus}-System™	AMERSHAM PHARMACIA BIOTECH
Gel Blotting Paper	SCHLEICHER & SCHUELL
Heparin Sepharose CL-6B	AMERSHAM PHARMACIA BIOTECH
Imaging plate BAS-IP MS 2325	FUJIFILM
Imidazole	MERCK
Immobilon™-P PVDF-Membrane	MILLIPORE
Malic acid	ICN
NAD	ICN
NADH	ICN
Ni-NTA Agarose	QIAGEN
Oxalacetic acid	ICN
PMSF	SIGMA

Precision Protein Standards™	BIO-RAD
Primers	MWG-BIOTECH
5´ IRD800-labelled primers	MWG-BIOTECH
Pwo-Polymerase	BOEHRINGER MANNHEIM
Restriction enzymes	INVITROGEN
T4-DNA-Ligase	PROMEGA
TEMED	INVITROGEN
Tissue Culture Test Plates 96flat/F	TPP
t RNA	SIGMA
Trans-label™	ICN
Tween 20	VWR-INTERNATIONAL
[α-^{32}P]-UTP	ICN, AMERSHAM PHARMACIA BIOTECH
X-ray films	AMERSHAM PHARMACIA BIOTECH
Zymolyase 20T	ICN

2.1.3 Kits

Jetquick PCR Purification Spin Kit	GENOMED
Jetquick Gel Extraction Spin Kit	GENOMED
Jetquick Plasmid Miniprep Spin Kit	GENOMED
Protein Assay	BIO-RAD
Riboprobe® Combination System-SP6/T7	PROMEGA
Thermo-Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP	AMERSHAM PHARMACIA BIOTECH

2.1.4 Antibodies

	Dilution in 1 x TBS-T with 5 % (w/v) skimmed milk powder
Mouse-Anti-Cox3p (MOLECULAR PROBES)	1:1000
Mouse-Anti-HA (BOEHRINGER MANNHEIM)	1:1000
Rabbit-Anti-Aac1p*	1:1000
Rabbit-Anti-Idhp*	1:1000
Sheep-Anti-mouse IgG-HRP (AMERSHAM PHARMACIA BIOTECH)	1:5000
Donkey-Anti-rabbit IgG-HRP (AMERSHAM PHARMACIA BIOTECH)	1:5000

Antibodies marked with (*) were kindly provided by H. van der Spek, Amsterdam.

2.1.5 Strains

2.1.5.1 *Escherichia coli* (*E. coli*)

Strain	Genotype	Reference
DH5α	Φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, □(*lacZYA-argF*)U169	Hanahan, 1983

2.1.5.2 *Saccharomyces cerevisiae* (*S. cerevisiae*)

Strain	Genotype	Reference
MMYO11	*MATα, ade2-1, can1-100, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1*	McCammon *et al.*, 1990
I2ΔHL	*MATα, ade2-1, can1-100, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1, idh2::HIS3*	Przybyla-Zawislak *et al.*, 1999
ΔIDH1L	*MATα, ade2-1, can1-100, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1, idh1::LEU2*	Przybyla-Zawislak *et al.*, 1999
I2ΔCIT1L	*MATα, ade2-1, can1-100, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1, idh2::HIS3, cit1::LEU2*	Przybyla-Zawislak *et al.*, 1999
I2ΔMDH1L	*MATα, ade2-1, can1-100, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1, , idh2::HIS3, mdh1::URA3*	Przybyla-Zawislak *et al.*, 1999
CIT1L	*MATα, ade2-1, can1-100, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1, cit1::LEU2*	Przybyla-Zawislak *et al.*, 1999
MDH1L	*MATα, ade2-1, can1-100, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1, mdh1::URA3*	Przybyla-Zawislak *et al.*, 1999

Y05376	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, cit1::kanMX4*	EUROSCARF
Y04934	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, mdh1::kanMX4*	EUROSCARF

2.1.6 Media

2.1.6.1 *E. coli*-media

LB	1.0 % (w/v) Tryptone 0.5 % (w/v) Yeast extract 0.5 % (w/v) NaCl 2.0 % (w/v) Agar (for LB-Plates)
Marker selection	100 µg/ml Ampicillin

2.1.6.2 *S. cerevisiae*-media

YPAce	1.0 % (w/v) Yeast extract 2.0 % (w/v) Peptone 2.0 % (w/v) Potassium phosphate 0.003 % (w/v) Adenine sulphate; pH6.0 2.0 % (w/v) Agar (for YP-Plates)
YPD	1.0 % (w/v) Yeast extract 2.0 % (w/v) Peptone 2.0 % (w/v) Glucose 2.0 % (w/v) Agar (for YP-Plates)
YPGal	1.0 % (w/v) Yeast extract 2.0 % (w/v) Peptone 2.0 % (w/v) Galactose 2.0 % (w/v) Agar (forYP-Plates)
YPGly	1.0 % (w/v) Yeast extract 2.0 % (w/v) Peptone 2.0 % (v/v) Glycerol 2.0 % (w/v) Agar (for YP-Plates)
WO	1.7 g/l Yeast nitrogen base 5.0 g/l Ammonium sulphate 2.0 % (w/v) Glucose 2.0 % (w/v) Agar (for WO-Plates)

2.1.6.3 Buffers

TBE	90 mM Tris 90 mM Boric acid 2.5 mM EDTA
TBS	137 mM NaCl 20 mM Tris-HCl (pH 7.4)
TBS-T	1 x TBS 0.1 % (v/v) Tween 20
PBS	140 mM NaCl 2.7 mM KCl 10.1 mM Na_2HPO_4 1.8 mM KH_2PO_4

2.1.6.4 Solutions for Bradford assay

Bradford solution	0.01 % (w/v) Coomassie® Brilliant blue G250 5 % (v/v) Ethanol 10 % (v/v) H_3PO_4

2.1.6.5 Solutions for SDS-PAGE

Running gel (12.5 %)	375 mM Tris-HCl, pH 8.8 12.5 % (w/v) Acrylamide 0.33 % (w/v) Bisacrylamide 0.1 % (w/v) SDS 0.1 % (w/v) Ammonium persulfate 0.1 % (v/v) TEMED
Stacking gel	125 mM Tris-HCl, pH 6.8 4 % (w/v) Acrylamide 0.1 % (w/v) Bisacrylamide 0.1 % (w/v) SDS 0.1 % (w/v) Ammonium persulfate 0.1 % (v/v) TEMED
Running buffer	25 mM Tris 192 mM Glycine 0.1 % (w/v) SDS
6 x loading buffer	300 mM Tris-HCl, pH 6.8 30 % (w/v) Glycerol 10 % (w/v) SDS 0.1 % (w/v) Bromphenol blue 600 mM DTT or 5 % ß-mercaptoethanol (freshly added)

Coomassie blue staining	42 % (v/v) Methanol 17 % (v/v) Acetic acid 0.1 % (w/v) Coomassie® Brilliant blue G250
Destaining solution	30 % (v/v) Methanol 7 % (v/v) Acetic acid

2.1.6.6 Solutions for silver staining

Fixation	50 % (v/v) Ethanol 5 % (v/v) Acetic acid
Washing	50 % (v/v) Methanol
Sensitizer	0.02 % (w/v) Sodium thiosulfate
Staining solution	0.1 % (w/v) $AgNO_3$
Developer	0.04 % (v/v) Formaldehyde 2 % (w/v) Na_2CO_3
Stop solution	5 % (v/v) Acetic acid

2.1.6.7 Solutions for Western blot

Transfer buffer	192 mM Glycine 25 mM Tris 5.0 % (v/v) Methanol 0.1 % (w/v) SDS
Ponceau S staining	0.5 % (w/v) Ponceau S 1 % (v/v) Acetic acid
Blocking solution	1 x TBS-T 5 % (w/v) skimmed milk powder

2.1.6.8 Solutions for band shift assay

Non-denaturing polyacrylamide gel (4 %)	4 % (w/v) Acrylamide 0.21 % (w/v) Bisacrylamide 1 x TBE 0.06 % (w/v) Ammonium persulfate 0.25 % (v/v) TEMED
Layermix	0.1 % (w/v) Bromphenolblue 10 % (v/v) Glycerol
10 x Binding buffer	100 mM Tris-HCl, pH 7.5 1 mM EDTA 30 mM $MgCl_2$ 500 mM NaCl 50 % (v/v) Glycerol 70 mM ß-mercaptoethanol 8 mM PMSF

2.1.7 Vectors

Vector	Genetic marker	Reference
pGH1	Amp^r, *URA3, CEN-ARS, TEF*-promoter, MCS, GFP-ORF, HA-Epitope	Mumberg *et al.*, 1995; Kruckeberg *et al.*, 1999
pGH2	Amp^r, *URA3, 2 µ, TEF*-promoter, MCS, GFP-ORF, HA-Epitope	Mumberg *et al.*, 1995; Kruckeberg *et al.*, 1999
pGH7	Amp^r, *LEU2, CEN-ARS, TEF*-promoter, MCS, GFP-ORF, HA-Epitope	Mumberg *et al.*, 1995; Kruckeberg *et al.*, 1999
pGH8	Amp^r, *LEU2, 2 µ, TEF*-promoter, MCS, GFP-ORF, HA-Epitope	Mumberg *et al.*, 1995; Kruckeberg *et al.*, 1999
YCplac33	Amp^r, *URA3, CEN-ARS, lacZ'*	Gietz and Sugino, 1988

Table 3. Vectors used for the plasmids construction.
The pGH1/2 vectors are modified versions of p41/42 TEF (Mumberg *et al.*, 1995) containing GFP (derived from the plasmid pGFP1 (Kruckeberg *et al.*, 1999)) and the HA-Epitope. The pGH7/8 vectors are similiar modified versions of p415/p425TEF.

2.1.8 Recombinant plasmids

Plasmid	Insert	Reference
pCOX2Δ9	ORF coding for 5`-UTR and coding region of *COX2*(-54 to +756)	Papadopoulou *et al.*, 1990
pCOBHE	ORF coding for 5`-UTR of *COB*(-243 to -26)	Dekker *et al.*, 1992
Yep352-Idh1p^{S92A}	ORF coding for Idh1p(S92A)	Cupp and McAlister-Henn, 1993
Yep352-Idh1p$^{K182L; Y184N}$	ORF coding for Idh1p(K182L, Y184N)	Siep, 2001

2.1.9 Primers

Primer	Sequence (5‘ → 3‘)	Enzyme site
1	GGGG*TTAATTAA***ATGTCAGCGATATTATCAACAAC**	*Pac*I
2	CCCG*GCGGCCGCCGGCGCGCC***GTTCTTACTTTCGATTTTCTTTAC**	*Not*I, *Asc*I
3	GGGG*TTAATTAA***ATGTTGTCAAGAGTAGCTAAACG**	*Pac*I
4	CCCG*GCGGCCGCCGGCGCGCC***TTTACTAGCAACAAAGTTGACACC**	*Not*I, *Asc*I
5	*CGCGCCGGCGGCCGCT***CATCATCATCATCATCATTAA***GG*	*Not*I
6	*CGCGCC***TTAATGATGATGATGATGATG**A*GCGGCCGCCGG*	*Not*I
uni-21*	**TGTAAAACGACGGCCAGT**	
rev-29*	**CAGGAAACAGCTATGACC**	

Table 4. Primers used for the plasmids construction and for sequencing.
The enzyme recognition sites are indicated in italic. The primer annealing regions are in bold, (*) indicates 5’ IRD800 labelled sequencing primers.

2.1.10 Recombinant plasmids of these work

Plasmid	Primers	Template	Protein
pGH1-Cit1p-GFP-HA	1, 2	yeast cDNA	Cit1p
pGH1-Cit1p-HA		pGH1-Cit1p-GFP -HA	Cit1p
pGH1-Cit1p-His_6	5, 6	pGH1-Cit1p-HA	Cit1p
pGH7-Mdh1p-GFP-HA	3, 4	yeast cDNA	Mdh1p
pGH7-Mdh1p-HA		pGH7-Mdh1p-GFP-HA	Mdh1p
pGH7-Mdh1p-His_6	5, 6	pGH7-Mdh1p-HA	Mdh1p
YCplac33-Idh1p^{S92A}		Yep352-Idh1p^{S92A}	Idh1p^{S92A}
YCplac33-Idh1p$^{K182L; Y184N}$		Yep352- Idh1p$^{K182L; Y184N}$	Idh1p$^{K182L; Y184N}$

Table 5. List of the plasmids constructed in this work.
The name of the plasmids are indicated on the left and the resulting protein on the right. The primers and the template used in the PCR or in the further cloning strategy are indicated.

2.2 Methods

2.2.1 Plasmids

The oligonucleotide primers used for PCRs are listed in Table 2. The combination of primers and the created constructs are listed in Table 3. The annealing temperature (T_a) of the primers was calculated according to the following formula (MWG-BIOTECH):

$T_a = 69.4°C + 0.41 * (\%\ of\ Primer\ GC) - 650/Primer\ length$

The following reaction mixture was used:

	Final concentration
Template DNA	1 ng/µl
Upstream primer (100 pmol/µl)	1 pmol/µl
Downstream primer (100 pmol/µl)	1 pmol/µl
dNTP mix (10 mM of each dNTP)	200µM of each dNTP
PCR puffer (10 x)	1 x
Pwo Polymerase (5 units/µl)	2 units
Total volume	**100 µl**

The following PCR program was used:

General PCR

5 min 95°C	Initial denaturation	1 x
45 sec 94°C 45 sec T_a + 5°C 1 min 72°C	Denaturation Annealing Elongation	5 x
45 sec 94°C 45 sec T_a 2 min 72°C	Denaturation Annealing Elongation	25 x
5 min 72°C	Final elongation	1 x

Standard techniques were used for restriction endonuclease analysis of DNA, ligation of DNA fragments, transformations and recovery of plasmid DNA from *E. coli* (Sambrook *et al.*, 1989). Purification of PCR products and plasmids was carried out with the kits listed in section 2.1.3.

A plasmid containing the active site mutant Idh1p^{S92A}, in which the serine at position 92 is changed into an alanine was generously provided by Lee McAlister-Henn (Texas, USA). The mutant gene coding for Idh1p$^{K182L;\ Y184N}$ which is deficient in mitochondrial RNA- binding because of the substitution of the residues lysine at position 182 and tyrosine at position 184 into leucine and asparagines was obtained from Sandra Elzinga (Amsterdam, Netherlands). Constructs YCplac33 Idh1p^{S92A} and YCplac33 Idh1p$^{K182L;\ Y184N}$ were derived by subcloning the according *Xba*I fragments containing the complete coding region of *IDH1* into YCplac33.

Plasmids pGH1-Cit1p-GFP-HA and pGH7-Mdh1p-GFP-HA, in which the yeast genes are fused C-terminal to the *GFP* gene and the HA-epitope, were obtained by cloning PCR products into the pGH1 or the pGH7 vector.

The vectors pGH1-Cit1p-HA and pGH7-Mdh1p-HA were constructed from the pGH1-Cit1p-GFP-HA and pGH7-Mdh1p-GFP-HA vectors by removing the *GFP* gene with the enzyme *Asc*I and religation of the resulting *Asc*I digested plasmids.

The constructs pGH1-Cit1p-HA and pGH7-Mdh1p-HA were used to tag *CIT1* and *MDH1* at their C-termini with 6 histidine residues. To create the C-terminal fusion the oligonucleotides no. 5 and 6 which are coding for 6 histidine residues including a stop signal and creating sticky ends of an *Asc*I-site were annealed. The plasmids pGH1-Cit1p-His_6 and pGH7-Mdh1p-His_6 were obtained by cloning of the annealing product into the *Asc*I-site of the respective constructs.

2.2.2 Sequencing

DNA sequences were determined by the dideoxy chain termination method of Sanger (Sanger *et al.*, 1977) using 5´ IRD800-labelled primers and the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP as described by the manufacturer and the LICOR DNA sequencer. Primers used for sequencing are listed in Table 2. The annealing temperature of the primers was calculated according the following formula (MWG-BIOTECH):

$$T_a = 69.4°C + 0.41 * (\%\ \text{of Primer GC}) - 650/\text{Primer length} + 3°C$$

2.2.3 Transformation of yeast cells

Yeast cells were transformed using the one-step procedure (Chen *et al.*, 1992).

2.2.4 Isolation of yeast mitochondria

2.2.4.1 Large scale preparation of mitochondria (modified procedure of Grivell , 1971)

Yeasts were grown to stationary phase in 3 l YPGal medium. Cells were harvested by centrifugation at 3000 x g for 5 minutes. After washing with water cells were resuspended in 2 ml/g wet weight DTT buffer and incubated at 30°C for 20 min. Cells were washed with 1.2 M sorbitol followed by suspension in 7 ml/g wet weight zymolyase buffer. The cells were incubated at 30°C with gentle shaking until spheroblasts were formed (about 30 min). Spheroblasts were then washed with 1.2 M sorbitol, suspended in 7 ml/g wet weight breaking buffer and lysed in a Dounce homogeniser. Unbroken cells and the cell debris were removed by centrifugation of the lysate at 3000 x g for 5 min at 4°C. Mitochondria were pelleted by centrifugation at 13000 x g for 30 min at 4°C, suspended in a small volume of SEM buffer and stored at -70°C.

DTT buffer	100 mM $TrisSo_4$, pH9.4 10 mM DTT
Zymolyase buffer	1.2 M Sorbitol 20 mM Potassium phosphate buffer, pH 7.4 2 mg/g wet weight Zymolyase 20T
Breaking buffer	0.6 M Sorbitol 10 mM Tris-HCl, pH 7.4 1 mM EDTA 0.2 % BSA (fatty acid free) 1 mM PMSF (freshly added)
SEM buffer	250 mM Saccharose 1 mM EDTA 10 mM MOPS, pH7.2 with KOH

2.2.4.2 Preparation of mitochondria (modified procedure of McKee, 1984)

The harvested and washed cells of 500 ml cultures were incubated in DTT buffer (5 ml/g wet weight) at 30°C for 20 min, washed with water and treated with zymolyase in spheroblast buffer (2 ml/g wet weight). After washing the spheroblasts were suspended in pottering buffer (2.5 ml/g wet weight) and lysed by using a Dounce homogeniser. Cell debris were removed by centrifugation at 3000 x g for 3 min at 4°C. The mitochondria were then pelleted, resuspended in SEM buffer and stored at -70°C.

DTT buffer	100 mM Tris, pH9.0 2.5 mM DTT
Spheroblast buffer	1.35 M Sorbitol 100 mM EDTA, pH 7.4 1 mg/g wet weight Zymolyase 20T
Pottering buffer	0.6 M Mannitol 1 mM EDTA, pH 6.7

2.2.5 Purification of mitochondrial proteins

For the preparation of mitochondrial extracts, frozen mitochondria from large scale preparations were thawed, pelleted, suspended in 10 ml of column buffer and broken by sonication for 4 x 15 sec. The lysate was applied to a Blue Sepharose column which had been equilibrated with column buffer. Proteins were eluted using a step gradient of 100-200-500 mM KCl in column buffer. Fractions were dialysed over night against dialysis buffer and concentrated by using Centricon® YM-10. The 0.2 M KCl Blue Sepharose protein fraction containing the RNA-binding factor(s) was adapted for about 1 hour to 50 mM KCl and then incubated 1 hour at 4°C with Heparin Sepharose equilibrated with column buffer. The Heparin Sepharose was pelleted by centrifugation 5 min at 500 x g and washed with column buffer. Proteins were eluted using a step gradient of 100-200-300-500 mM KCl in column buffer. The fractions were dialysed 4 hours at 4°C against dialysis buffer and assayed for RNA- binding activity. Proteins were analysed by SDS-PAGE and silver staining.

Column buffer	50 mM KCl 20 mM Tris, pH 7.5 0.1 mM EDTA 10 % Glycerol 1 mM PMSF 6 mM ß-mercaptoethanol
Dialysis buffer	20 mM KCl 20 mM Tris, pH 7.5 0.1 mM EDTA 10 % Glycerol 1 mM PMSF 6 mM ß-mercaptoethanol

2.2.6 Affinity purification of recombinant mitochondrial proteins

Yeast null mutant strains transformed with the plasmid pGH1-Cit1-6xHis or pGH7-Mdh1-6xHis carrying the coding region for the genes of interest and a His-Tag were inoculated from an overnight selection culture and grown at 30°C in YPGal medium. Mitochondria were prepared, suspended in buffer A and lysed by sonication for 4 x 15 sec. The lysate was incubated with Ni-NTA agarose which had been equilibrated with buffer A. The Ni-NTA agarose was pelleted by centrifugation 5 min at 500 x g and washed with buffer A. Proteins were eluted using imidazole as competitor in buffer B, assayed for enzymatic and RNA-binding activity and stored at – 70°C. The purification grade was analysed by SDS-PAGE and silver staining.

buffer A	20 mM Imidazol 300 mM NaCl 50 mM NaH_2PO_4, pH 7.4 0.1 mM EDTA 10 % Glycerol 1 mM PMSF 6 mM ß-mercaptoethanol
buffer B	200 mM Imidazol 300 mM NaCl 50 mM NaH_2PO_4, pH 7.4 0.1 mM EDTA 10 % Glycerol 1 mM PMSF 6 mM ß-mercaptoethanol

2.2.7 Determination of Citrate synthase activity (Srere *et al.*, 1963)

Citrate synthase activity was measured in 1 ml 100 mM Tris-HCl buffer, pH 8.1, 0.1 mM DTNB (Ellman´s reagent), 0.3 mM acetyl-CoA and 0.5 mM oxalacetic acid. Assays were performed at room temperature and initiated by the addition of oxalacetic acid. The reaction was follwed spectrophotometrically at 412 nm controlling a possible acetyl-CoA deacylase activity and after the addition of oxalacetic acid measuring the increase in absorbance resulting from the formed mercaptide ion (acetyl-CoA + oxalacetic acid → citrate + CoASH; CoASH + DTNB → CoA-TNB + TNB^-). One unit forms one micromole of CoASH per minute under the described conditions. Specific activity is expressed as enzyme unit(s) per milligram of protein.

The enzymatic activity was calculated according to the following formula:

$$\text{Units} = \frac{\Delta A_{412}/\text{min}}{\varepsilon_{412}(\text{DTNB}) * \text{d}}$$

The specific enzymatic activity was calculated according to the following formula:

$$\text{Units/mg} = \frac{\Delta A_{412}/\text{min}}{\varepsilon_{412}(\text{DTNB}) * \text{d} * \text{mg enzyme/ml reaction mixture}}$$

ε_{412} (DTNB) = 13.6 mM^{-1} cm^{-1}

d = 1 cm

2.2.8 Determination of Malate dehydrogenase activity (McAlister-Henn and Thompson, 1987)

Malate dehydrogenase activity was measured in 1 ml 50 mM potassium phosphate buffer, pH 7.5, 0.12 mM NADH (freshly prepared) and 0.33 mM oxalacetic acid (freshly prepared). Assays were performed at room temperature and initiated by the addition of partial or total purified protein. The reaction was followed spectrophotometrically at 340 nm measuring the decrease in absorbance resulting from the oxidation of NADH. One unit oxidizes one micromole of NADH per minute under the described conditions. Specific activity is expressed as enzyme unit(s) per milligram of protein.

The enzymatic activity was calculated according to the following formula:

$$\text{Units} = \frac{\Delta A_{340}/\text{min}}{\varepsilon_{340}(\text{NADH}) * \text{d}}$$

The specific enzymatic activity was calculated according to the following formula:

$$\text{Units/mg} = \frac{\Delta A_{340}/\text{min}}{\varepsilon_{340}(\text{NADH}) * \text{d} * \text{mg enzyme/ml reaction mixture}}$$

ε_{340} (NADH) = 6.22 mM^{-1} cm^{-1}

d = 1 cm

2.2.9 *In vitro* transcription

Templates for run-off *in vitro* transcription were produced by digestion of respective plasmids with suitable restriction endonucleases (schematically represented in Fig. 2). After purification of digested DNA, run-off transcripts of pCOX2Δ9**Rsa*I, pCOX2Δ9**Hind*III and pCOBHE**Eco*RI were made by incubation of 250 ng of template DNA for 90 min at 37°C in the presence (for direct binding studies) or absence (for competition assays) of [α-^{32}P]-UTP according to standard procedures (Melton, 1984). T7 *COX2* transcripts contain either the 5'-leader (-54 to + 50) of *COX2* or the 5'-leader of *COX2* plus the corresponding coding region (-54 to +756) from *S. cerevisiae*. SP6 transcripts contain the 5'-leader of COB (-343 to -26) from *S. cerevisiae*. All three transcripts start with polylinker sequences derived from the corresponding transcription vector. Unincorporated nucleotides were removed by purification over a 1 ml Sephadex-G50 column. Yields of labelled transcripts were calculated from the incorporation of [α-^{32}P]-UTP into RNA. Their integrity was analysed on 6 % polyacryl-amide/7 M urea gels and visualised by autoradiography.

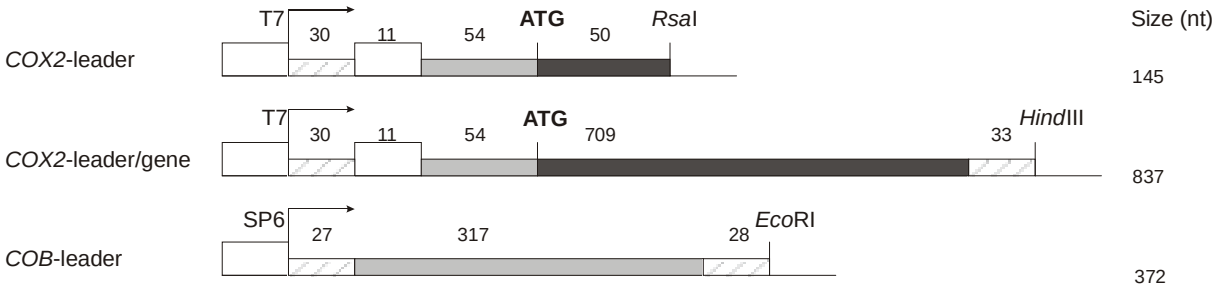


Fig. 2: DNA templates used for *in vitro* transcription.
Right-angled arrows denote the start and direction of transcription. Polylinker sequences (hatched lines); nucleotides upstream of the transcriptional start (white bars), leaders (lightly-shaded boxes), initiation codon (ATG) and coding regions (black boxes) are indicated. Templates were linearised by cleavage at the restriction sites shown.

2.2.10 *In vitro* RNA-protein binding assay

The RNA-protein binding assay was performed according to the protocol developed for the analysis of NAD^+-depending Isocitrate dehydrogenase to mitochondrial RNAs (Papadopoulou *et al.*, 1990).

For a standard experiment 1 µg of purified protein was mixed with 0.5 up to 3 ng [α-^{32}P]-UTP labelled RNA (100 cpm) in a final volume of 30 µl binding buffer and incubated for 15 min at 30°C. Calf liver competitor RNA (1 µg) was added in the reaction mixture in order to minimise the non-specific association of proteins with RNA. For competition assays different amounts of molar excess of unlabelled RNA probes were preincubated with the protein for 5 min at 30 ° C prior to the addition of the specific labelled probe and further incubation for 15 min. After incubation, the protein-RNA complexes were resolved by electrophoresis through 4 % non-denaturing polyacrylamide gels run at 180 V for 2.5 hours at 4°C using 1 x TBE as gel and running buffer. The gels were dried and visualised by autoradiography.

Binding buffer	50 mM NaCl 10 mM Tris, pH 7.5 0.1 mM EDTA 5 % Glycerol 0.8 mM PMSF 7 mM ß-mercaptoethanol 3 mM $MgCl_2$

2.2.11 *In vitro* translation

For efficient translation, isolated mitochondria were resuspended in 0.6 M mannitol to a final concentration of 3 mg protein per ml and incubated in optimised protein synthesising medium in the presence of 8 µl/ml Trans-Label^{TM} at 30°C for 1 hour. 250 µl samples were taken at 30 min and 60 min. An excess of cold methionine (0.2 M) was added to start the chase and samples were taken after 60 min. The samples were pelleted by centrifugation for 2 min at 15000 x g, washed with 0.6 M mannitol/1 mM EDTA (pH 6.7), separated on a SDS-PAGE, blotted on a PVDF membrane and analysed by immunodetection.

optimised protein synthesising medium	600 mM Mannitol 150 mM KCl 15 mM KH_2PO_4 12.5 mM $MgSO_4$ 4 mM ATP 0.5 mM GTP 5 mM α-Ketoglutarate 5 mM Phosphoenolpyruvate 10 units/ml Pyruvate kinase 0.1 mM amino acids (minus methionine) 20 mM Tris 3 mg/ml Bovine serum albumin, pH7.2

2.2.12 Determination of the protein concentration

The protein concentration was determined using the protein assay as described by the supplier or by Bradford (Bradford, 1976).

2.2.13 Western blot analysis

Proteins were separated on a 12.5 % polyacrylamide gel in the presence of SDS. Proteins were transferred to a PVDF membrane using the tank blotting method for 1 h at 4°C at 1,5 mA/cm^2. Membranes were blocked over night at 4°C or 1 hour at room temperature with 5 % (w/v) non-fat dry milk in TBS-T. Membranes were probed with polyclonal or monoclonal antisera for 1 hour at room temperature and then washed three times for 10 minutes in TBS-T. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies were used as secondary antibodies. The secondary antibody was added to the membranes for 30 minutes at room temperature and the membranes were again washed three times for

10 minutes in TBS-T. Antigen-antibody complexes were visualised by enhanced chemiluminescence (ECL plus).

2.2.14 Blue native gel-electrophoresis

The blue native gel-electrophoresis was performed as described by Schägger and Jagow (1991). Solubilisation of mitochondria was achieved by using 2 % lauryl-maltoside.

3 RESULTS

3.1 Characterisation of Idh1 mutant proteins

Idhp is a member of a still growing family of enzymes with an additional RNA-binding property. To get more information about the mode of action of that bifunctional protein and to understand the link between dehydrogenase and RNA-binding activities an enzymatically deficient isocitrate binding site mutant, IDH1^{S92A} (Cupp and McAlister-Henn, 1993), and a mutant deficient in RNA-binding, IDH1$^{K182L/Y184N}$ (Siep, 2001), were used.

3.1.1 Complementation analysis of *idh1* mutants

A functional isocitrate binding site of Idh1p is necessary for respiratory growth.

Mutant genes encoding Idh1p^{S92A} and Idh1p$^{K182L/Y184N}$ were expressed under control of the authentic promoter in the single-copy vector YCplac33. Each of the proteins was expressed in the respiratory deficient Δ*idh1* strain ΔIDH1L and the transformants were tested for their ability to restore normal growth on the non-fermentable carbon source glycerol. The wild type strain MMYO11 was used as a positive control. The *idh1* null mutant strain and the *idh1* null mutant strain transformed with the vector YCplac33 were used as negative controls. The mutant deficient in mitochondrial mRNA-binding did grow on glycerol while the enzymatic mutant Idh1p^{S92A} did not (Fig. 3). This is contrary to the observation that active site mutants of Idh1p are able to confer normal respiratory growth (Lin *et al.*, 2001) and indicates that the enzymatic function of Idhp is required for growth on that non-fermentable carbon source. To test whether instability of Idh1p^{S92A} is responsible for the *idh1* null mutant phenotype the steady state concentration was analysed.

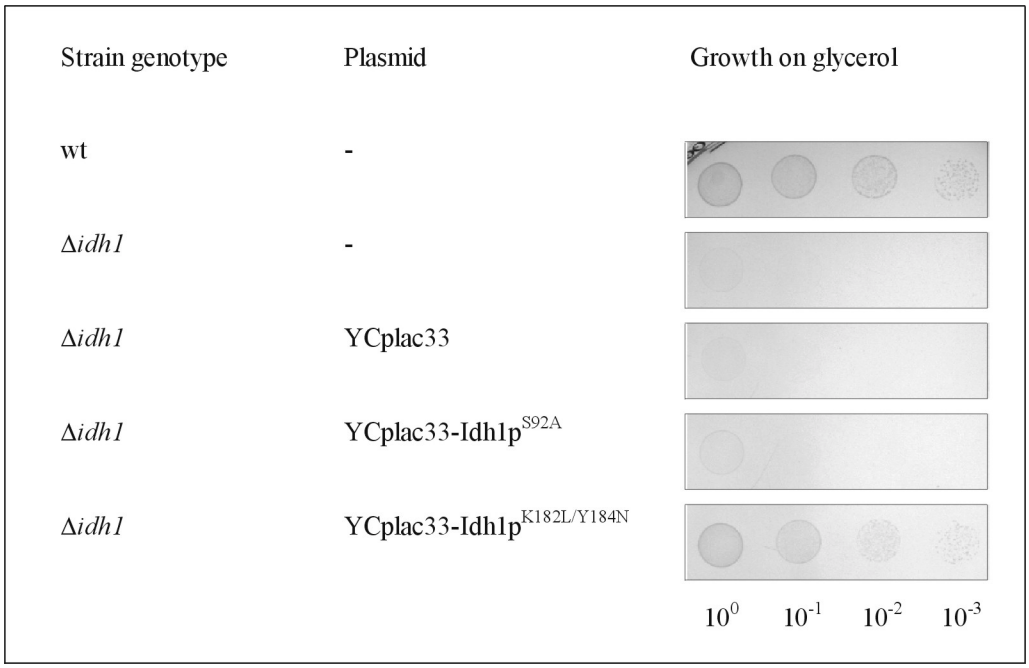


Fig. 3: Complementation behaviour of Idh1 mutant proteins in strain ΔIDH1L
Strain ΔIDH1L was transformed with YCplac33 as a negative control or with plasmids encoding Idh1p^{S92A} or Idh1p$^{K182L/Y184}$. Strain MMYO11 served as a positive control. All strains were grown overnight in liquid minimal medium supplemented with selective amino acids and resuspended to 2 units OD_{600}. This suspension was serially diluted in 10-fold steps. For each strain, 5 µl from each dilution were spotted onto rich medium with glycerol as a carbon source (YPGly). Growth of cultures on glycerol medium was monitored after 3 days of incubation at 30°C.

3.1.2 Influence of *idh1* mutations on protein stability

The Idh1 mutant proteins show reduced steady state levels.

In order to compare the stability of the wild type protein and the mutant proteins mitochondria of ΔIDH1L transformants expressing either Idh1p^{S92A} or Idh1p$^{K182L/Y184N}$ were isolated. The wild type strain MMYO11 was used as a positive control. ΔIDH1L bearing or not bearing the vector YCplac33 was used as negative control. Equal amounts of mitochondrial proteins were separated on a 15 % SDS-PAGE, transferred to PVDF membrane and probed with a polyclonal Idh antibody. As the *IDH1* genes are expressed from their own promoter their expression should be similiar. Fig. 4 shows that only the wild type strain (lane 1) contains equal amounts of the Idh subunits while the ΔIDH1L transformants expressing Idh1p^{S92A} (lane 4) and Idh1p$^{K182L/Y184N}$ (lane 5) contain less Idh1p (upper band) and a wild type like amount of Idh2p (lower band). Other weak signals which were also detected in the controls probably result from crossreactions with the antibody. To consider the fact that Idhp is an abundant protein, comprising at least 0.4 % of total mitochondrial protein (Dekker *et al.*,

1991), it could be ne-cessary to increase the expression level. Since Idh1p^{S92A} and Idh1p$^{K182L/Y184N}$ expressed under control of the authentic promoter in multi-copy vectors also show reduced steady state levels (data not shown) the impaired function of Idh1p seems to be responsible for that behaviour.

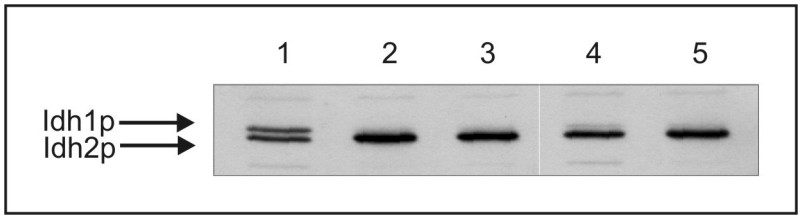


Fig. 4: Stability of Idh1 mutant proteins in strain ΔIDH1L
Mitochondria were isolated from MMYO11 (1), strain ΔIDH1L (2) and strain ΔIDH1L transformed with the vector YCplac33 (3) or with plasmids encoding Idh1p^{S92A} (4) or Idh1p$^{K182L/Y184N}$ (5). Western blot analysis was performed with a polyclonal antiserum directed against Idhp.

3.1.3 Effect of *idh1* mutations on mitochondrial translation products

The absence of Idh1p leads to reduced steady state levels of Cox1p, Cox2p and Cox3p and of Cyt*b* (de Jong *et al.*, 2000). Since the effect is most pronounced with Cox3p the analysis of the influence of *idh1* mutations on mitochondrially encoded proteins was focused on Cox3p. Mitochondria of the wild type, the *idh1* null mutant and ΔIDH1L transformants expressing one of the *idh1* mutant proteins were prepared for an *in vitro* translation assay. Equal amounts of mitochondria were incubated with protein synthesising medium containing L-[^{35}S] labelled methionine as described in Materials and Methods (2.2.11). After 30 and 60 min the pulse-labelled samples were taken. The labelling reaction was then stopped by addition of an excess of unlabelled methionine to follow the turnover of the synthesised proteins and the chase samples were taken after 60 min. After gel-electrophoresis and Western blotting the labelled proteins were visualised using a phosphorimager (Fig. 5A). Contrary to the expectations that the synthesis of Cox1p, 2 and 3 is strongly increased in the absence of Idh1p and that the increase of newly synthesised proteins in the *idh1* null mutant is accompanied by an enhanced turnover (de Jong *et al.*, 2000) the results presented in Fig. 5A show wild type like synthesis (Fig. 4A; lanes 30/60 min pulse of wt and Δ*idh1*) and turnover (Fig. 5A; lanes 60 min chase wt and Δ*idh1*). Similarly, increase in protein synthesis (Fig. 5A; lanes 30/60 min pulse of idh1^{S92A} and idh1$^{K182L/Y184N}$) and enhanced turnover (Fig. 5A; lanes 60 min chase of idh1^{S92A}

and idh1$^{K182L/Y184N}$) cannot be observed in *idh1* transformants. The mitochondrial translation activity of strains MMYO11 and ΔIDH1L was lower, perhaps because these strains are not isogenic. The effect of *idh1* mutations on steady-state levels of cytochrome *c* oxidase were analysed with antisera directed against subunit Cox3p. Equal protein amounts in all lanes were monitored by using a polyclonal antibody for the ATP-ADP carrier Aac1p (Fig. 5B). The enzymatic mutant of Idh1p (panel c) shows decreased amounts of Cox3p and Cox3p instability like a null mutant (panel b). The RNA-binding deficient mutant (panel d) turned out to have wild type level (panel a) of Cox3p.

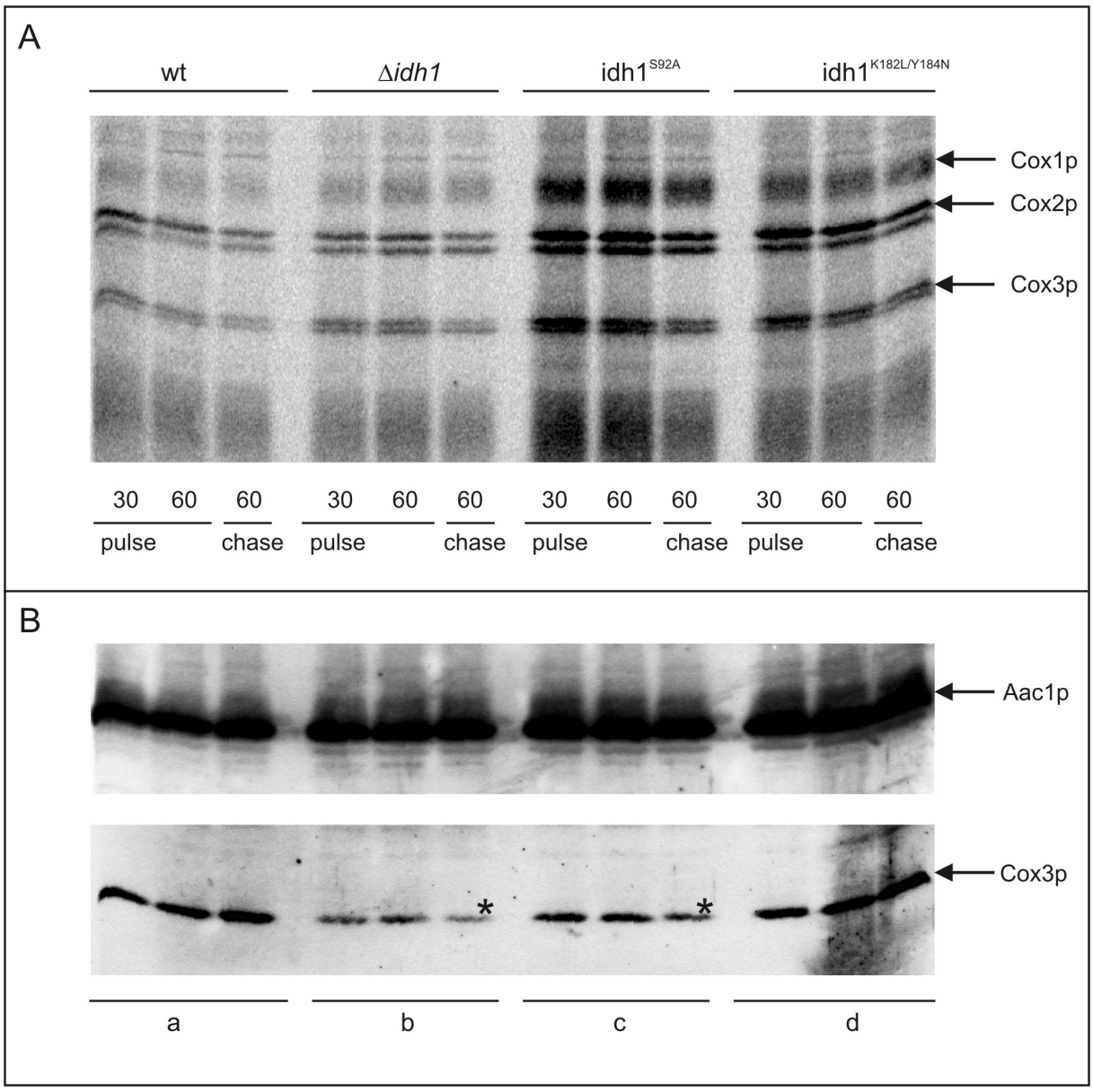


Fig. 5: Translation in mitochondria from *idh1* mutants compared to wild type
Mitochondria from MMYO11 (wt), strain ΔIDH1L (Δ*idh1*) and strain ΔIDH1L transformed with plasmids encoding Idh1p^{S92A} (idh1^{S92A}) or Idh1p$^{K182L/Y184N}$ (idh1 $^{K182L/Y184N}$) were isolated and used for a pulse-chase labelling experiment. Proteins were pulse-labelled with L-[^{35}S] methionine for 30 and 60 min. After addition of a large excess of unlabelled methionine, mitochondria were chased for 60 min. Samples were taken at the indicated time points and resolved on 12.5 % SDS-PAGE.
(**A**) After transfer to a PVDF membrane bands were visualised using a phosphorimager. Positions of the mitochondrial products are indicated on the right.
(**B**) Cox3p was detected with a monoclonal antibody and as a control an antibody against Aac1p was used. Cox3p bands which are marked with asterisks indicate an enhanced turnover.

3.2 Evidence for occurrence of Idhp in a high molecular weight complex

Idhp is in a high molecular weight complex.

On one hand it seems conceivable that Idhp as an important part of the Krebs cycle interacts with other enzymes to form a metabolon (Robinson *et al.*, 1987). On the other hand Idhp is involved in the regulation of mitochondrial translation by binding to mRNAs and therefore might be connected with proteins of the mitochondrial translation machinery.

To detect possible interactions the molecular organization of Idhp was investigated by the Blue Native gel-electrophoresis (Schägger *et al.*, 1994). Conditions that allow detection of several other mitochondrial complexes and intermediates in the assembly of cytochrome *c* oxidase (Nijtmans *et al.*, 1998) were used. Application of this technique to mitochondrial extracts of strain MMYO11 allowed the immunological detection of Idhp in a high molecular weight complex (Fig. 6). This complex possesses a higher molecular weight than cytochrome *c* oxidase (about 250 kDa) which was identified with antibodies directed against Cox3p. The exact size of the complex cannot be calculated from this experiment. Since both subunits were detectable (the original of Fig. 5 reveals two spots at the position of the Idhp signal) and since it is known that the protein is acting as an octamer the signal could reflect the molecular mass (about 320 kDa) of this multimeric form. Whether this complex consists of multimers of Idhp or of Idhp in association with other proteins remains to be elucidated.

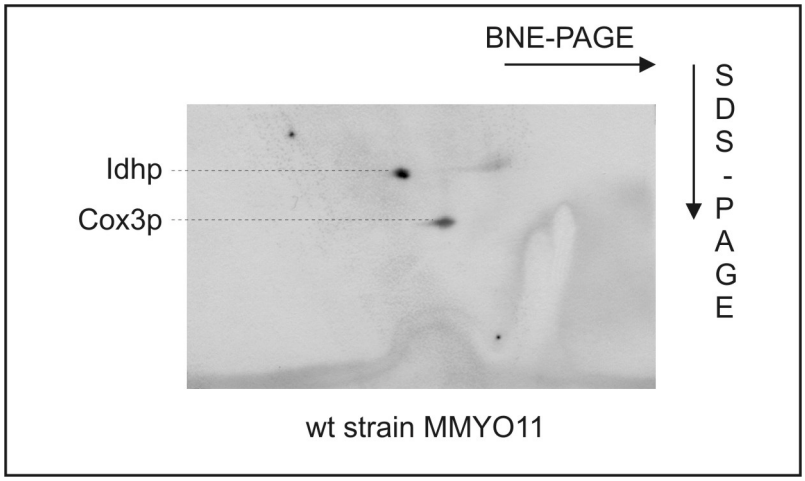


Fig. 6: 2D PAGE of mitochondria from the wild type strain MMYO11
Mitochondrial proteins of strain MMYO11 were analysed in a 2D BNE (first dimension)-SDS (second dimension) gel system. After Western blotting, successive immunodetections with antibodies against Cox3p and against Idhp were performed. Arrows indicate the direction of BNE- and SDS-PAGE electrophoresis. The positions of Idhp and Cox3p are indicated on the left.

3.3 Identification of RNA-binding activities in *idh2* null mutant strains

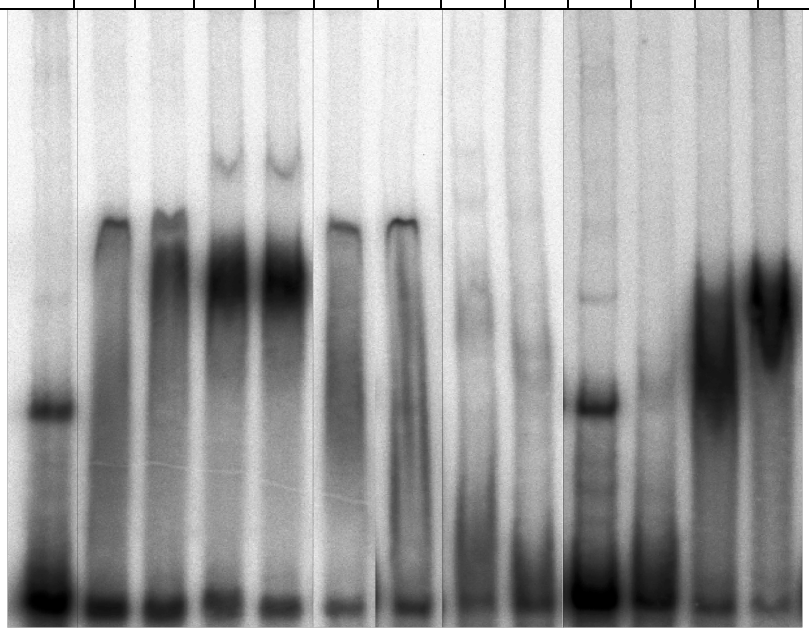
Cit1p and Mdh1p are RNA-binding proteins.

To identify and characterise proteins, which might be involved in the regulation of mitochondrial translation, the gel retardation assay established for Idhp was used (Papadopoulou *et al.*, 1990). Since the Krebs cycle enzyme Idhp binds with high affinity to mitochondrial mRNAs and could possibly cover other binding activities, mitochondrial extracts from an *idh2* null mutant strain were compared in retardation experiments with extracts from *idh2cit1* and *idh2mdh1* null mutants. Deletions of *cit1* and *mdh1* encoding citrate synthase1 and malate dehydrogenase1 were chosen because preliminary results indicated that Cit1p and Mdh1p are able to bind mitochondrial mRNAs. Surprisingly *cit1* or *mdh1* mutations, occurring in the absence of either one or both Idh subunits, have been reported to suppress the *idh* null mutation phenotype (Gadde and McCammon, 1997). Since Cit1p and Mdh1p are members of the same biochemical pathway as isocitrate dehydrogenase this observation is unexpected and could hint at an additional function of Cit1p and Mdh1p, eventually with respect to the RNA-binding function of Idhp . Whether Cit1p and/or Mdh1p are involved in RNA-binding was tested in the following way: mitochondrial proteins of the different *idh* null mutant strains were purified as described in Materials and Methods (2.1.5). The fractions eluted from Heparin Sepharose by increasing KCl concentrations were incubated with [α-^{32}P] labelled RNA which contains the complete 5'-untranslated region and

part of the coding sequence of *COX2* (termed as *COX2* leader RNA) (Fig. 7A). The *COX2* leader is the shortest mitochondrial leader and therefore especially suited for direct protein-RNA interaction studies by band-shift assays. The integrity of *COX2* leader RNA was analysed on a 6 % polyacrylamide-7M urea gel and visualized by autoradiography (data not shown). Probably due to alternative secondary structures in addition to a major band several additional bands can be detected (lane 1). All fractions from the Δ*idh2* null mutant strain I2ΔHL show binding to the *COX2* mRNA (lanes 2-5). Whereas the first two fractions of the Δ*idh2*Δc*it1* double null mutant strain I2ΔCIT1L (lanes 6 and 7) still show a RNA band-shift similiar to that of first fractions of I2ΔHL (lanes 2 and 3), no or strongly decreased RNA-binding was observed in the respective fractions of the Δ*idh2*Δ*mdh1* double null mutant strain I2ΔMDH1L (lanes 10 and 11). Comparison of the band-shifts of the last two fractions of each strain reveals similar RNA-binding activities of I2ΔHL (lanes 4 and 5) and I2ΔMDH1L (lanes 12 and 13) but significantly reduced RNA-binding activity in the respective I2ΔCIT1L fractions (lane 8 and 9) suggesting that the RNA-binding may be caused by Cit1p. To determine whether the RNA-binding properties correlate with the enzymatic activities of Cit1p and Mdh1p enzyme assays of all Heparin Sepharose fractions were performed. Highest specific activities of Mdh1p were found in the first two fractions and highest specific activities of Cit1p were found in the last two fractions. Obviously the RNA-binding activities correlate with the enzymatic activities (Fig. 7B). Therefore it seems likely that the observed protein-RNA complex formation of *idh2* null mutant lysates is mediated by Cit1p and/or by Mdh1p. As malate dehydrogenase activity is also present in the last two elution fractions of I2ΔCIT1L Mdh1p might be responsible for the residual band shift. However the presence of other binding proteins cannot be excluded.

Complexes were also formed with the doubled concentration of competitor RNA (2 µg) and at 4, 30 and 37°C (data not shown), indicating that the *in vitro* RNA-binding is specific. To gain insight into the nature of the interactions involved in the binding of Cit1p and Mdh1p to *COX2* leader RNA, the effect of increasing amounts of KCl and Heparin on Cit1p/Mdh1p-*COX2* RNA-binding was determined (data not shown). Formation of both complexes proved to be resistant to high KCl concentrations (200 mM) indicating that ionic interactions do not play a prominent role in the binding of Cit1p and Mdh1p. The polyanion Heparin interfered with the formation of both complexes, but only at moderate to high concentrations (600-6000 nM). The ability of Heparin to completely inhibit Cit1p/Mdh1p-*COX2* RNA-binding may reflect that hydrophobic interactions are involved in these protein-RNA interactions.

A	1	2	3	4	5	6	7	8	9	10	11	12	13
fraction from I2ΔHL (KCl in mM)		100	200	300	500								
fraction from I2ΔCIT1L (KCl in mM)						100	200	300	500				
fraction from I2ΔMDH1L (KCl in mM)										100	200	300	500



B		2	3	4	5	6	7	8	9	10	11	12	13
fraction from I2ΔHL (KCl in mM)		100	200	300	500								
fraction from I2ΔCIT1L (KCl in mM)						100	200	300	500				
fraction from I2ΔMDH1L (KCl in mM)										100	200	300	500

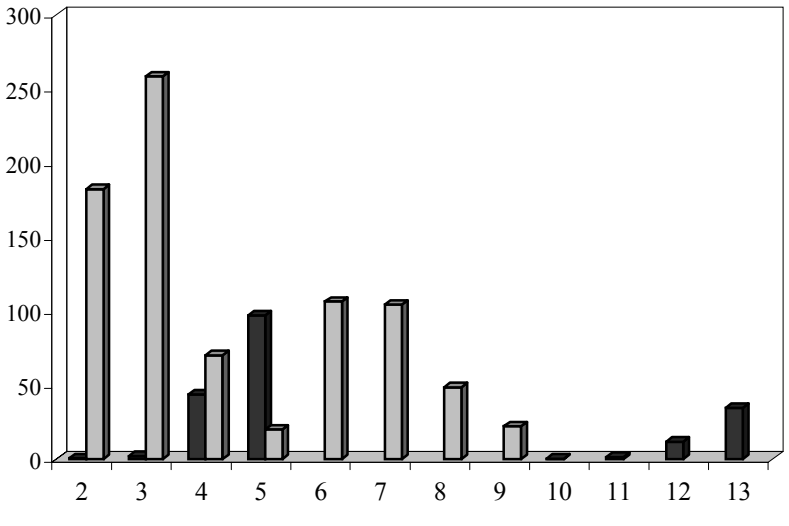


Fig. 7: *COX2* RNA-binding activities *in vitro* correspond to enzymatic activities of Cit1p and Mdh1p
Binding assays were performed using [α-^{32}P] labelled *COX2* leader RNA (ca. 2 ng) made *in vitro* by a T7 transcription system and mitochondrial protein fractions (1 µg) eluted from Heparin Sepharose as described in Materials and Methods. After mobility shift assay, radioactive material was visualized by autoradiography.
(**A**) Lane 1, free RNA; lanes 2–5, RNA with protein fractions of I2ΔHL, lanes 6-9, RNA with protein fractions of I2ΔCIT1L, lanes 10-13, RNA with protein fractions of I2ΔMDH1L.
(**B**) All fractions were assayed for citrate synthase (light columns) and/or malate dehydrogenase (dark columns) activity. Specific enzymatic activities are mean values of 3 independent measurements.

3.4 Effect of *cit1* and *mdh1* null mutations on mitochondrial translation products

Gel retardation assays indicated that the Krebs cycle enzymes Cit1p and Mdh1p are able to bind *COX2* leader RNA independent of Idhp and therefore might also be involved in regulation of mitochondrial translation. To assess the effect of Cit1p and Mdh1p on mitochondrial translation, newly synthesised proteins in mitochondria isolated from the wild type, an *idh2* null mutant, and from *cit1* and *mdh1* null mutants were compared in a pulse-labelling experiment (Fig. 8A). Samples were taken after 30 and 60 min labelling using L-[^{35}S] methionine and after 60 min chase in the presence of unlabelled methionine. Equal amounts of mitochondrial proteins were separated by SDS-PAGE and then blotted to a PVDF membrane. Labelled translation products were analysed by phosphorimager scannning. The results presented in Fig. 8A show that in the absence of Cit1p (panel Δ*cit1*) and Mdh1p (panel Δ*mdh1*), the synthesis of mitochondrially encoded proteins seems to be not influenced. Synthesis of Cox2p and Cox3p (Fig. 8A; lanes 30/60 min pulse of wt, Δ*cit1* and Δ*mdh1*) and their stability (Fig. 8A; lanes 60 min chase of wt, Δ*cit1* and Δ*mdh1*) are comparable to wild type. The increase of Cox3p synthesis and its enhanced degradation in the *idh2* null mutant (panel Δ*idh2*) is hardly detectable. Since not only synthesis and turnover but also the steady-state levels of mitochondrial proteins are affected in mitochondria lacking the Krebs cycle enzyme Idhp, the steady-state level of Cox3p was analysed, too (Fig. 8B). The concentration of the ATP-ADP carrier Aac1p, which was used as an control, shows in all lanes comparable protein concentrations (Fig. 8B). While Cox3p is hardly detectable in an *idh2* null mutant (panel b), strains lacking *cit1* (panel c) and *mdh1* (panel d) show wild type level (panel a). These data indicate that both Cit1p and Mdh1p are not directly involved in mitochondrial translation.

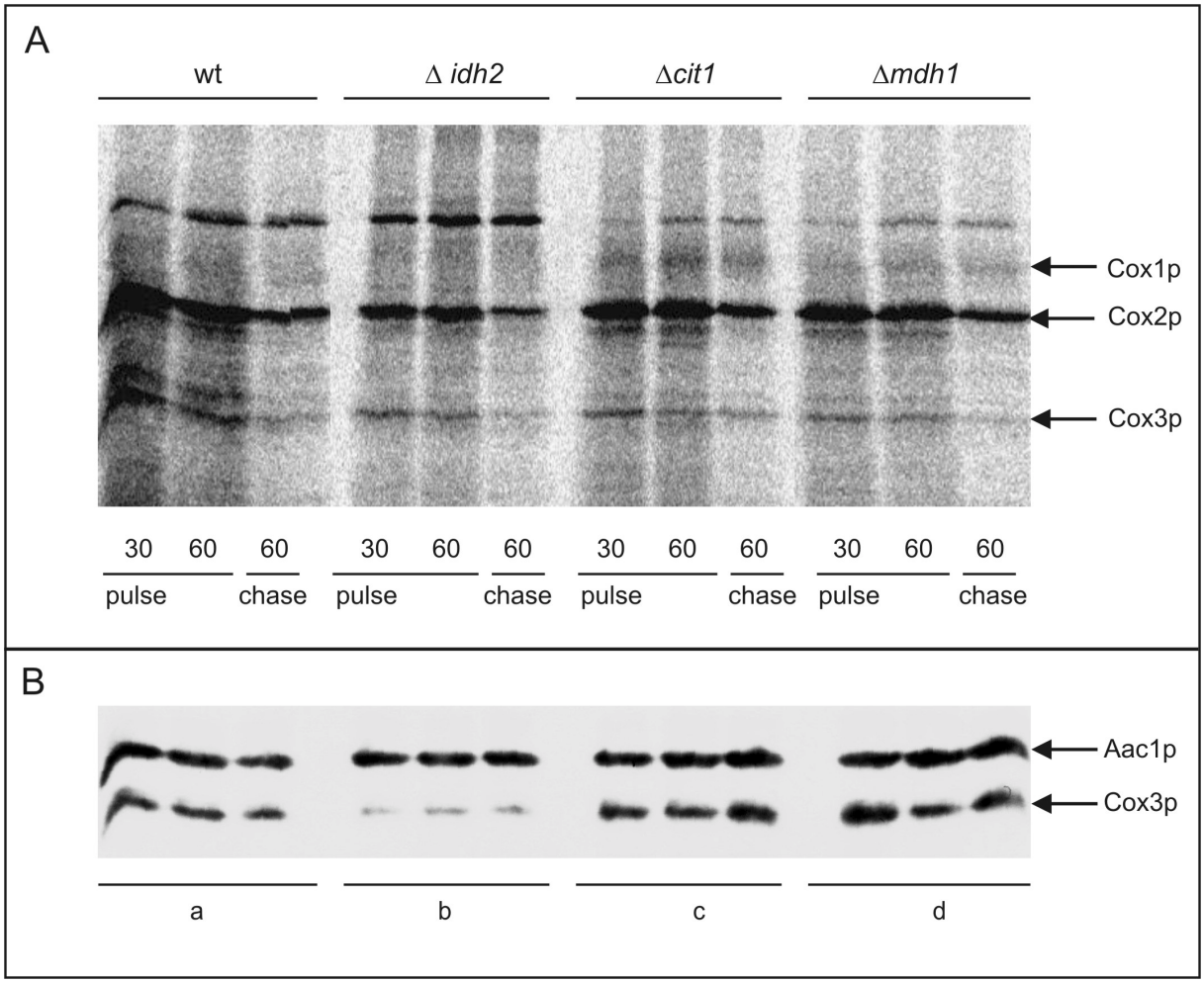


Fig. 8: Influence of null mutations of Krebs cycle enzymes on mitochondrial protein synthesis

Mitochondrial translation products of MMYO11 (wt), I2ΔHL (Δ*idh2*), ΔCIT1L (Δ*cit1*) and ΔMDH1L (Δ*mdh1*) were labelled with L-[^{35}S] methionine for 60 min, followed by the addition of an excess of unlabelled methionine and a 60 min chase. Samples were taken after 30 and 60 min labelling and after 60 min chase, separated by a 12.5 % SDS-PAGE and blotted on a membrane.

(**A**) Newly synthesised proteins were analysed by phospho-imaging. The cytochrome *c* oxidase subunits Cox1p, Cox2p and Cox3p are indicated on the right.

(**B**) Immunodetections were performed using a monoclonal antibody against Cox3p. As a control, the blot was incubated with an antibody against Aac1p.

3.5 Functional analysis of C-terminally tagged forms of Cit1p and Mdh1p

Since no antibodies directed against Cit1p and Mdh1p were available, epitope-tagged variants of the *CIT1* and *MDH1* genes were constructed. Tagging of these proteins could change their native structure and affect their enzymatic function, although so far such effects have not been reported The functionality of histidine-tagged Cit1p and Mdh1p was analysed by complementation analysis and enzyme assays. The recombinant proteins were isolated from yeast mitochondria using affinity chromatography with Ni-NTA and tested for their ability to bind to RNA. Because the His_6 antibodies also react with a number of proteins, which contain less than a series of six histidines, they cannot be used for immunoblots. Therefore in addition the HA-epitope was used and the participation of Cit1p and Mdh1p in high molecular weight complexes was analysed using protein-HA fusions.

3.5.1 Complementation analysis of Cit1p and Mdh1p

Tagging of Cit1p and Mdh1p does not affect the ability to complement the mutant growth phenotype.

The epitope tag HA was placed at the C-terminus of the investigated proteins (pGH1-Cit1p-HA; pGH7-Mdh1p-HA). To be able to purify Cit1p and Mdh1p from whole cell lysates in a one step procedure, the proteins were C-terminal fused to six histidine residues (pGH1-Cit1p-His_6; pGH7-Mdh1p-His_6). Mutants lacking Cit1p or Mdh1p are unable to grow on the non-fermentable carbon source acetate. Functionality of the fusion proteins was tested by transformation of the respective genes into ΔCIT1L or ΔMDH1L and analysis of the acetate growth characteristics of the resulting transformants. As a positive control the wild type strain MMYO11 was used. As negative controls the *cit1* and *mdh1* null mutant strains and the *cit1* and *mdh1* null mutant strains transformed with the vector pGH1 or pGH7 were used. As presented in Fig. 9 transformants containing one of the vectors do not restore growth on acetate, whereas transformants bearing the HA- or His_6-tagged genes do. This observation shows that the tagging of Cit1p and Mdh1p does not disturb their functionality.

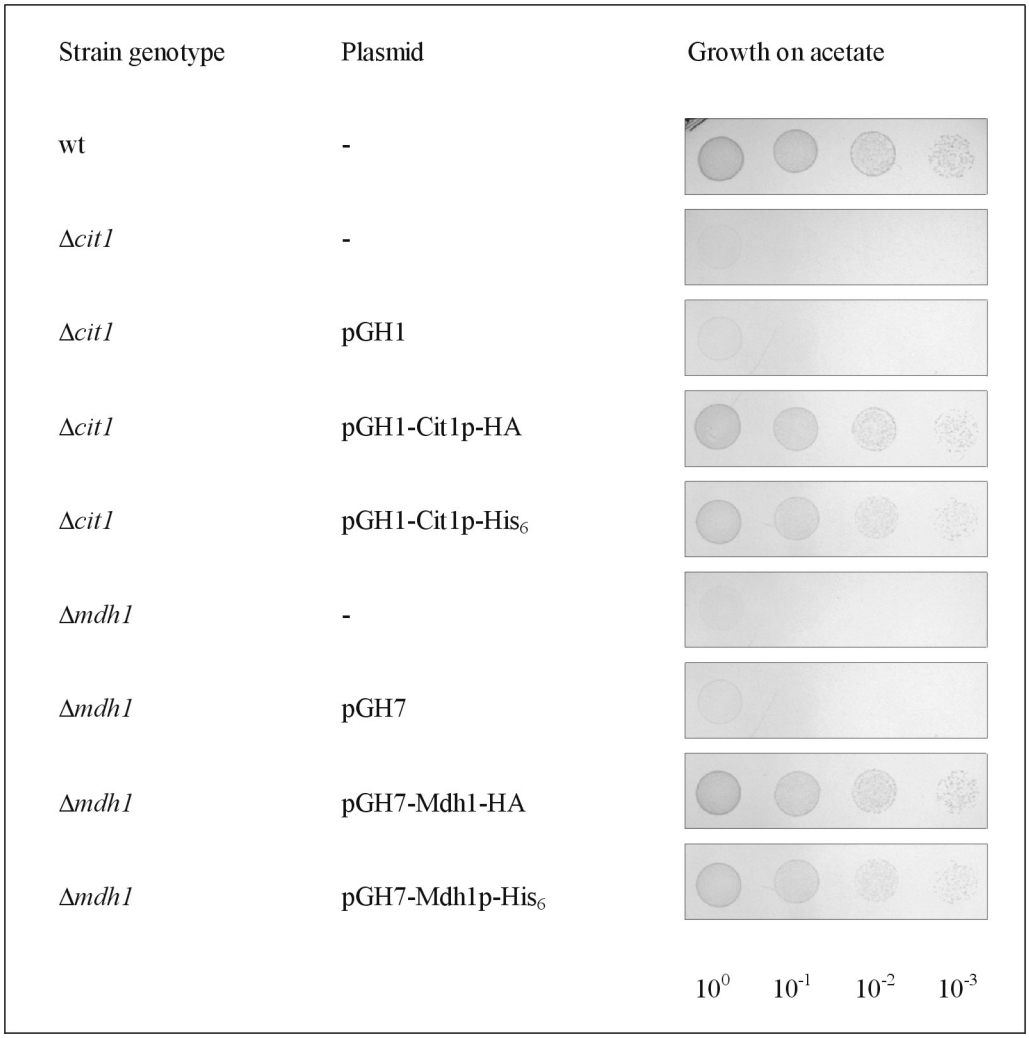


Fig. 9: Complementation of the acetate$^-$ phenotype of strains ΔCIT1L and ΔMDH1L
Strain ΔCIT1L was transformed with pGH1 as a negative control or with plasmids encoding Cit1p-HA or Cit1p-His_6. Strain ΔMDH1L was transformed with pGH7 as a negative control or with plasmids encoding Mdh1p-HA or Mdh1p-His_6. Strain MMYO11 served as a positive control. All strains were grown overnight in liquid minimal medium supplemented with selective amino acids and resuspended to 2 units OD_{600}. This suspension was serially diluted in 10-fold steps. For each strain, 5 µl from each dilution were spotted onto rich medium with acetate as a carbon source (YPAce). Growth of cultures on acetate medium was monitored after 3 days of incubation at 30°C.

3.5.2 Affinity purification of Cit1p and Mdh1p

Proteins, which were purified from whole cells disrupted with glass beads, showed an increased instability. Possibly not all proteases could be inactivated and caused the degradation. For that reason mitochondria of *cit1* or *mdh1* null mutant strains transformed with the plasmids pGH1-Cit1-His_6 or pGH7-Mdh1-His_6 were isolated, lysed and used for affinity purification. Western blot analysis (Fig. 10A) shows that both Cit1p-His_6 (49 kDa; lane 2) and Mdh1p-His_6 (33 kDa; lane 3) could successfully be isolated. The molecular mass

is consistent with that wild type protein plus additional six histidine residues. The purity of the recombinant proteins was high as deduced from silver staining of the gels. Whether the conditions of the affinity chromatography permit a copurification of interacting proteins was investigated by enzyme assays. Indeed, this approach revealed that the purified Cit1p-His_6 contains tiny amounts of Mdh1p and the purified Mdh1p-His_6 shows traces of Cit1p activity. Idh activity was not detected. To exclude the possibility of copurification the His-tagged construct of *CIT1* was transformed into the Δ*mdh1*-strain Y04934, while the His-tagged construct of *MDH1* was transformed into the Δ*cit1*-strain Y05376. The recombinant proteins were isolated from yeast mitochondria according to the protocol for affinity chromatography (2.2.6). Purity was examined by means of gel staining with silver nitrate (Fig. 10B). In contrast to the pre-vious purification two bands for each protein (lane 3; Cit1p-His_6 and lane 6; Mdh1p-His_6) were observed. Because Cit1p and Mdh1p are present *in vivo* as dimers (Musrati *et al.*, 1998; Reif *et al.*, 2001) the result probably reflects copurification of tagged and untagged proteins.

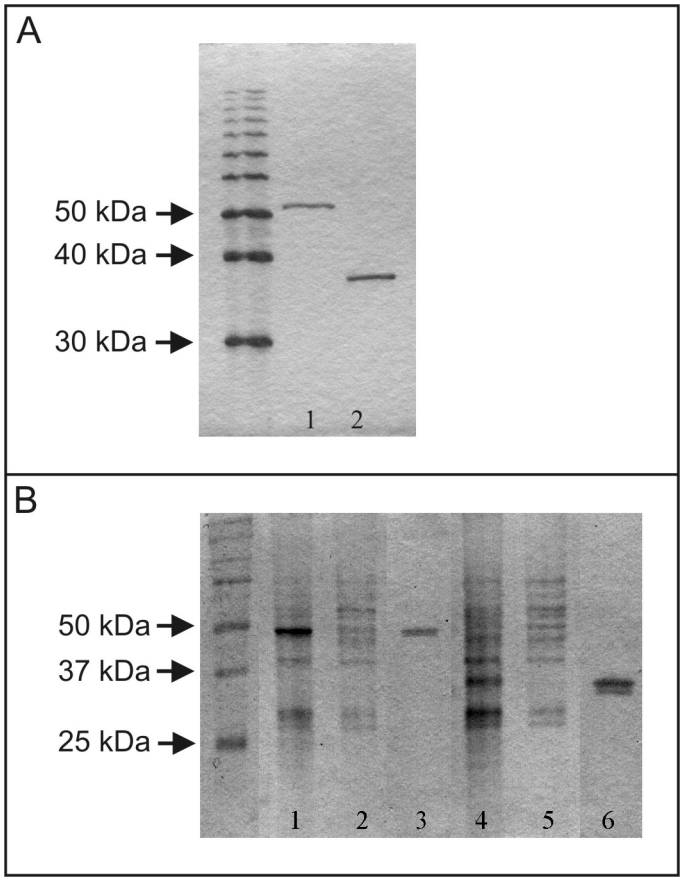


Fig. 10: Purification of C-terminally His-tagged Cit1p and Mdh1p from the corresponding null mutants
The C-terminally His-tagged forms of Cit1p and Mdh1p were affinity purified using chromatography on Ni-NTA and silver stained after electrophoresis on 12.5 % SDS polyacrylamide gel.
(**A**) Cit1p-His_6 and Mdh1p-His_6 were expressed in the corresponding null mutant strains ΔCIT1L and ΔMDH1L. Lane 1, purified Cit1p-His_6, lane 2, purified Mdh1p-His_6.
(**B**) Cit1p-His_6 was expressed in the Δ*mdh1*-strain Y04934 and the Mdh1p-His_6 was expressed in the Δ*cit1*-strain Y05376. Lane 1, mitochondrial lysate prior to incubation with Ni-NTA, lane 2, mitochondrial lysate after incubation with Ni-NTA, lane 3, purified Cit1p His_6, lane 4, mitochondrial lysate prior to incubation with Ni-NTA, lane 5, mitochondrial lysate after incubation with Ni-NTA, lane 6, purified Mdh1p His_6.

3.5.3 Enzymatic activity of Cit1p and Mdh1p

Tagging does not affect the catalytic activity of Cit1p and Mdh1p.

Cit1p and Mdh1p carry out catalytic functions in the Krebs cycle. To test whether epitope-tagged forms of these proteins act like wild type enzymes, recombinant Cit1p-His_6 and Mdh1p-His_6 were assayed for their enzymatic activity. The specific activities measured for the affinity-purified enzymes (Table 6) are similar to that obtained by conventional methods (Srere *et al.*, 1963; McAlister-Henn and Thompson, 1987). This result suggests that the fusion of six histidines does not interfere with the enzymatic function of Cit1p and Mdh1p.

Table 6: Specific enzymatic activities of purified enzymes
Enzymes were purified in form of His-tagged proteins. Cit1p was expressed in the Δ*mdh1*-strain Y04934, Mdh1p in the Δ*cit1*-strain Y05376. Citrate synthase or malate dehydrogenase activitiy was determined in triplicate.

Protein	Cit1p-His_6	Mdh1p-His_6
Specific activity (U/mg)	129.75	143.41

3.5.4 Analysis of protein-protein interactions of Cit1p and Mdh1p

Cit1p and Mdh1p do not interact with mitochondrial proteins.

To detect possible interactions between Krebs cycle enzymes or respiratory chain subunits and Cit1p and Mdh1p by 2D Blue Native gel-electrophoresis mitochondrial proteins from ΔCIT1L transformed with pGH1-Cit1p-HA, and from ΔMDH1L transformed with pGH7-Mdh1p-HA, were applied to a BN-PAGE in the first dimension and then separated by SDS-PAGE in a second dimension. Proteins were analysed by immunoblotting with monoclonal

antibodies against HA and Cox3p. The detection of Cit1p-HA (Fig. 11A) and Mdh1p-HA (Fig. 11B), respectively, revealed at the same position in the first dimension a strong signal and a number of further spots probably caused by degradation products. An additional weak signal for both proteins is observed. Dimerisation experiments of Cit1p showed that almost all monomers assemble into dimers (Reif *et al.*, 2001). Since Mdh1p forms dimers as well, the strong signal probably represents the dimeric form, while the weak signal might reflect the monomeric form. An additional signal (marked with an asterisk) results from a protein crossreacting with the HA-antibody. This protein is also detected in lysates lacking HA-epitope containing proteins. Cox3p indicates the position of the COX complex (about 250 kDa). Regarding the location of COX it can be assumed that Cit1p and Mdh1p are localised in a mitochondrial complex with a molecular weight lower than 250 kDa. However, existence of weak interactions with other proteins, which are disrupted by the extraction or separation techniques used, cannot be ruled out.

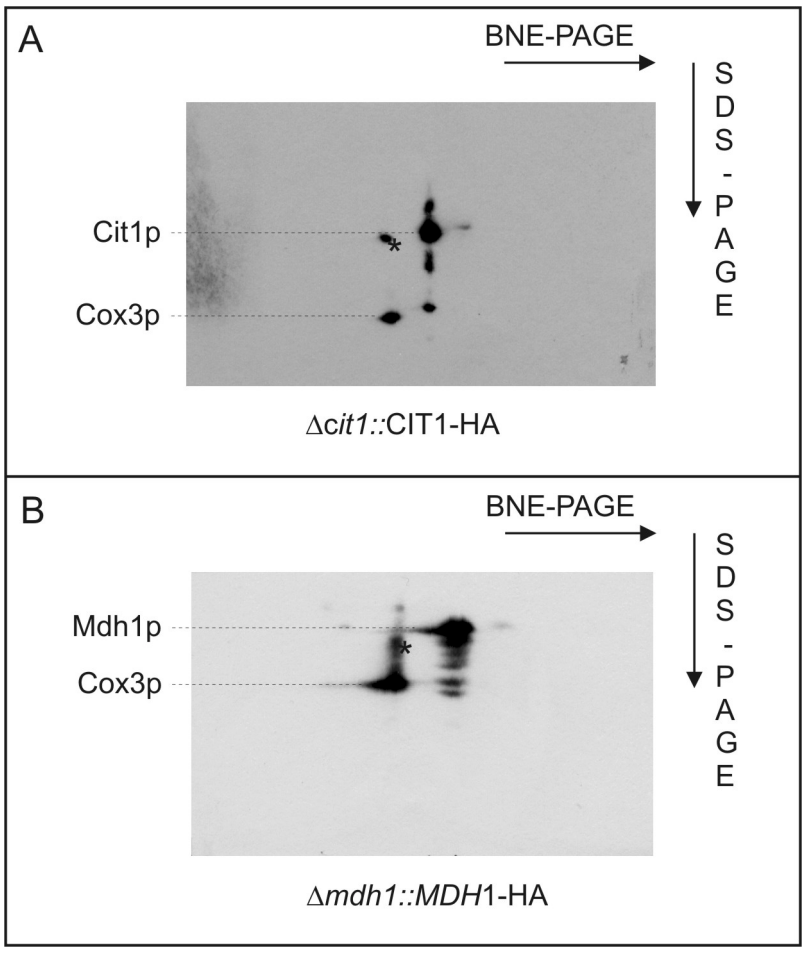


Fig. 11: Localisation of Cit1p and Mdh1p
Mitochondrial proteins of ΔCIT1L transformed with pGH1-Cit1p-HA (**A**) and of ΔMDH1L transformed with pGH7-Mdh1p-HA (**B**) were analysed in a 2D BNE (first dimension)-SDS (second dimension) gel system. After Western blotting, successive immunodetections with antibodies against the HA-Epitope and against Cox3p were performed. Arrows indicate the direction of BNE and SDS-PAGE electrophoresis. The positions of Cit1p-HA and Cox3p are indicated on the left. The crossreacting signal of the HA-antibody is marked with an asterisk.

3.5.5 Cit1p-His_6 and Mdh1p-His_6 bind to *COX2* leader RNA

His-tagged forms of Cit1p and Mdh1p bind to RNA.

As shown by complementation analysis (3.5.1) growth on acetate as sole carbon source was restored by transformation of the null mutants with pGH1-CIT1-His_6 or pGH7-MDH1-His_6. Obviously the histidine-tagged forms of Cit1p or Mdh1p perform normal metabolic functions *in vivo*. When Cit1p-His_6 and Mdh1p-His_6 were analysed for *in vitro* RNA-binding using the *COX2* leader probe, complexes were formed (Fig. 11, lanes 2 and 3). This suggests that the presence of the C-terminal His-tag does not interfere with RNA-binding. Whether the histidine residues by themselves mediate RNA-binding was controlled by His-tagged EGFP (enhanced green fluorescent protein), which is often used to investigate protein localisation (lane 4). At a protein concentration of 1 µg retarded bands which are comparable to those mediated by the untagged enzymes were observed. Although a clear retardation was achieved with a less amount of protein (data not shown) 1 µg of affinity purified protein was used in the following experiments. This concentration seems to be sufficient to saturate all RNA-binding sites because higher protein concentrations did not result in a increase in the observed band shift. His-tagged EGFP did not lead to retardation of the RNA.

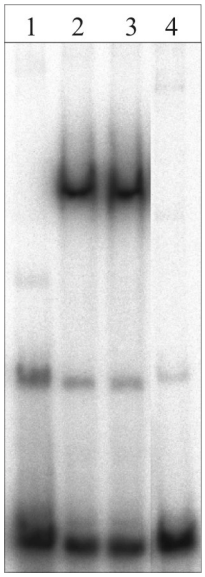


Fig. 11: RNA-binding analysis of histidine tagged proteins
RNA-binding activities of Cit1p-His_6 (lane 2) and Mdh1p-His_6 (lane 3) were assessed in a gel retardation assay with radiolabelled *COX2* leader RNA from *S. cerevisiae* (lane 1). RNA-protein complexes were separated in a 4 % native polyacrylamide gel and visualised by autoradiography. His-EGFP (lane 4) served as a negative control.

3.6 Specificity of Cit1p/Mdh1p RNA-binding

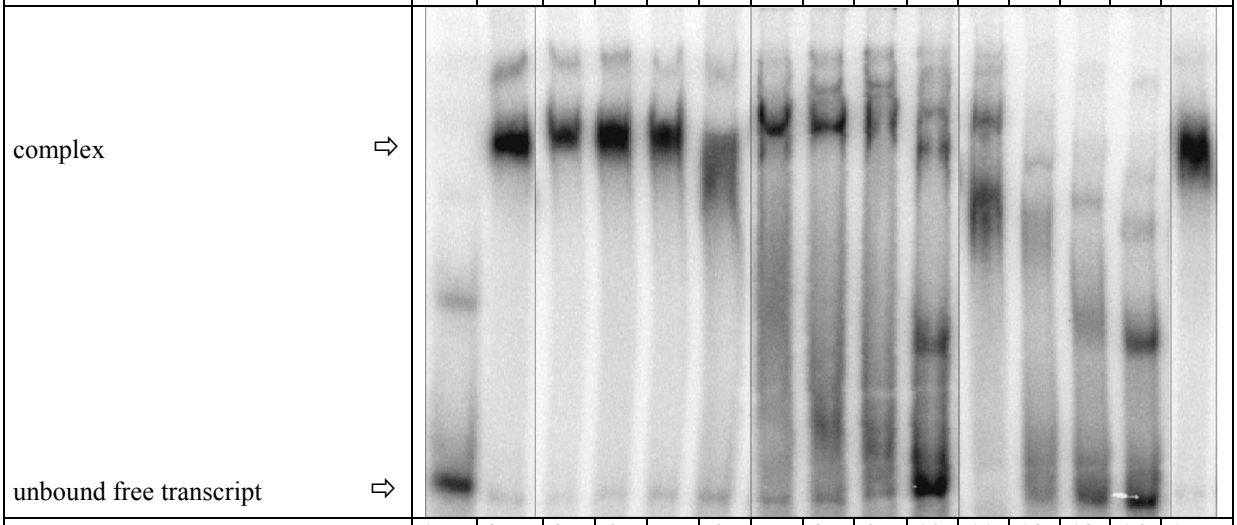
The secondary structure of mitochondrial mRNAs may define the target site of Cit1p and Mdh1p.

In the previous chapters it has been demonstrated that Cit1p and Mdh1p exhibit binding to an RNA encompassing the 5' untranslated leader and a small part of the coding region of *COX2*. To study the binding specificity of the proteins, the gel retardation assay with *COX2* transcript was performed in the presence of homologous and heterologous RNAs. As competitors the unlabelled transcripts of *COX2* leader (-54 to +50), *COB* leader (-343 to -26) and *COX2* gene including the leader (-54 to +756) from *S. cerevisiae* (Fig. 2 in Materials and methods) were used, tRNA from yeast served as unspecific competitor. The effect of the preincubation with the respective RNA on radiolabelled RNA-protein complex formation was determined with histidine-tagged proteins (Fig. 12). In the presence of increasing amounts of unlabelled *COX2* leader RNA with a constant amount of labelled *COX2* leader RNA the complexes were similarly diminished (lanes 3-6). Increasing molar excess of unlabelled *COB* leader RNA (lanes 7-10) caused a stronger competition than the addition of *COX2* leader RNA, while increasing molar amounts of *COX2* leader/gene RNA (lanes 11-14) caused the strongest competition. The protein binding to the labelled RNA was inhibited in the order: *COX2* leader/gene > *COB* leader > *COX2* leader RNA, when each compound was tested at 1, 5, 10

and 50-fold molar excess. These results indicate that Cit1p and Mdh1p are capable of interacting specifically with mitochondrial mRNAs. Since antisense transcripts made from *COB* leader RNA and from *COX2* leader/gene RNA have the same affinity for the proteins like the sense RNAs, Cit1p and Mdh1p seems to select a secondary structure motif that is common in both RNA species. These experiments also confirmed the findings with partially purified proteins (data not shown) that there is a specific interaction between the investigated Krebs cycle enzymes and mitochondrial RNAs. Since all transcripts have different sizes (from 104 nt for *COX2* leader to 810 nt for *COX2* leader/gene) and can presumably fold in different secondary structures, additional studies are needed to elucidate possible sequence/structure requirements for Cit1p and Mdh1p binding.

In contrast, preincubation with the non-specific competitor tRNA had no significant effect on the level of protein interaction with the *COX2* probe. A 1000-fold molar excess of tRNA referring to the *COX2* leader RNA slightly diminished the retardation of the RNA but did not compete (lanes 15).

A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Cox2 leader	/	/	1	5	10	50	/	/	/	/	/	/	/	/	/
Cob leader	/	/	/	/	/	/	1	5	10	50		/	/	/	/
Cox2 leader/gene	/	/	/	/	/	/	/	/	/	/	1	5	10	50	/
tRNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	1000



B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Cox2 leader	/	/	1	5	10	50	/	/	/	/	/	/	/	/	
Cob leader	/	/	/	/	/	/	1	5	10	50		/	/	/	
Cox2 leader/gene	/	/	/	/	/	/	/	/	/	/	1	5	10	50	
tRNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	1000

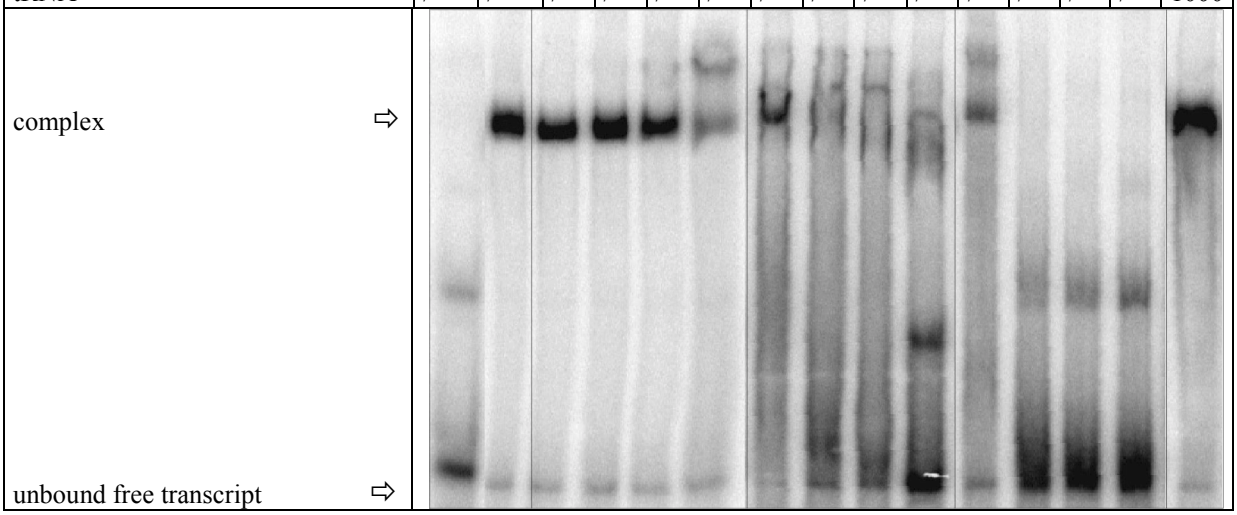


Fig. 12: Competition analysis of *COX2* RNA-binding by Cit1p-His$_6$ and Mdh1p-His$_6$ *in vitro*

Increasing amounts of unlabelled competitor RNAs were mixed with the histidine-tagged proteins prior to the addition of labelled *COX2* probe.

(**A**) Mobility shift of *COX2*-Cit1p-His$_6$. Lane 1, free RNA; lane 2, complex formation between *COX2* RNA and Cit1p-His$_6$, lane 3-6, competition with unlabelled *COX2* leader RNA, lane 7-10, competition with unlabelled *COB* leader RNA, lane 11-14, competition with unlabelled *COX2* leader/gene RNA, lane 15, competition with yeast tRNA. The molar excess of competitor over the labelled RNA is given.

(**B**) Mobility shift of *COX2*-Mdh1p-His$_6$. Lane 1, free RNA; lane 2, complex formation between *COX2* RNA and Mdh1p-His$_6$, lane 3-6, competition with unlabelled *COX2* leader RNA, lane 7-10, competition with unlabelled *COB* leader RNA, lane 11-14, competition with unlabelled *COX2* leader/gene RNA, lane 15, competition with yeast tRNA. The molar excess of competitor over the labelled RNA is given.

3.7 Influence of cofactors and substrates on Cit1p/Mdh1p RNA-binding

It has been shown for other RNA-binding proteins, for instance glyceraldehyde-3-phosphate dehydrogenase, catalase and glutamate dehydrogenase that the RNA-binding activity can be inhibited by cofactors or allosteric ligands (Nagy and Rigby, 1995; Clerch *et al.*, 1996; Bringaud *et al.*, 1997). Since both citrate synthase and malate dehydrogenase activities are regulated by several nucleotide effectors, whose levels fluctuate dependent on the energy state of the mitochondria, the influence of acetyl-CoA, AMP/ATP and NAD^+/NADH on the RNA-binding capacity of Cit1p or Mdh1p was investigated. The effect of an incubation with the substrates oxaloacetate, citrate or malate on RNA-binding activities of Cit1p or Mdh1p was also examined. The cofactors or substrates were added to the binding reaction in high molar excess (final concentrations indicated in Fig. 13 and 14). The effect on RNA-binding was assayed by mobility shift analysis. As shown in Fig. 13, a concentration of 5 mM of acetyl-CoA (lane 4) and ATP (lane 10) decreased Cit1p and Mdh1p binding of [α-^{32}P] labelled *COX2* leader, whereas a concentration of 5 mM of AMP (lane 7), NAD^+ (lane 13) and NADH (lane 16) did not affect the RNA-binding. An effect of the latter substances on complex formation can be observed when higher concentrations (50 mM) are used. Since these concentrations affect the mobility of free RNA as well, this effect is likely to be artefactual. Band shift analysis of Cit1p-His_6 and Mdh1p-His_6 in the presence of oxaloacetate (lanes 19), of citrate or malate (lanes 22) showed that binding is also inhibited at a concentration of 50 mM. In that case mobility of free RNA was not altered. These data indicate that the substrate acetyl-CoA and the cofactor ATP may regulate RNA-binding by both enzymes to the *COX2* leader. The specifity of these inhibitory effects on Cit1p/Mdh1p-RNA interactions was supported by the effect of acetyl-CoA and ATP on RNA-binding activities of partially purified proteins (data not shown). The other substrates or cofactors are probably not involved in the regulation of RNA-binding.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
acetyl-CoA	/	/	0.5	5	50	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
AMP	/	/	/	/	/	0.5	5	50	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
ATP	/	/	/	/	/	/	/	/	0.5	5	50	/	/	/	/	/	/	/	/	/	/	/	/
NAD^+	/	/	/	/	/	/	/	/	/	/	/	0.5	5	50	/	/	/	/	/	/	/	/	/
NADH	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.5	5	50	/	/	/	/	/	/
oxaloacetate	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.5	5	50	/	/	/
citrate	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.5	5	50

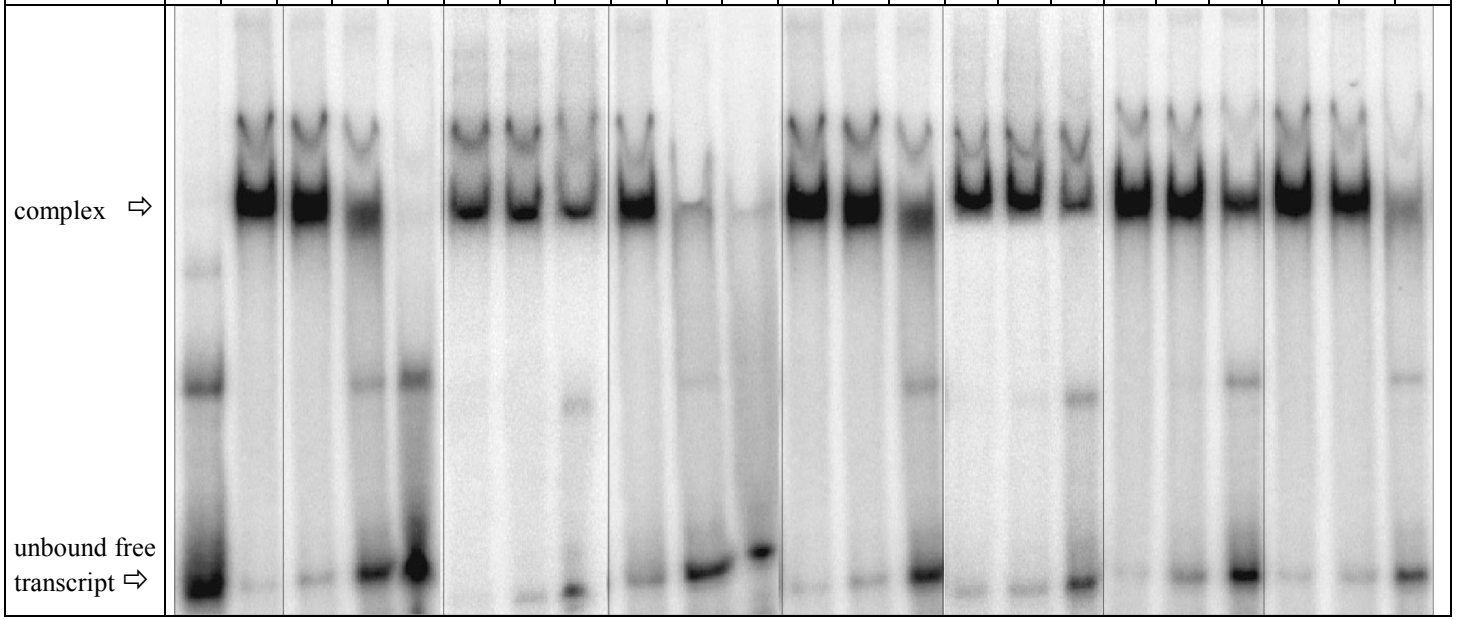


Fig. 13: Influence of various factors on RNA-binding of Cit1p
COX2 RNA was incubated without (lane 1) and with 1 µg of purified Cit1p-His_6 (lane 2). Lanes 3-23 acetyl-CoA, AMP, ATP, NAD^+, NADH, oxaloacetate or citrate were added to the binding reactions in the final concentrations as indicated in the figure (in mM).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
acetyl-CoA	/	/	0.5	5	50	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
AMP	/	/	/	/	/	0.5	5	50	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
ATP	/	/	/	/	/	/	/	/	0.5	5	50	/	/	/	/	/	/	/	/	/	/	/	/
NAD^+	/	/	/	/	/	/	/	/	/	/	/	0.5	5	50	/	/	/	/	/	/	/	/	/
NADH	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.5	5	50	/	/	/	/	/	/
oxaloacetate	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.5	5	50	/	/	/
malate	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.5	5	50

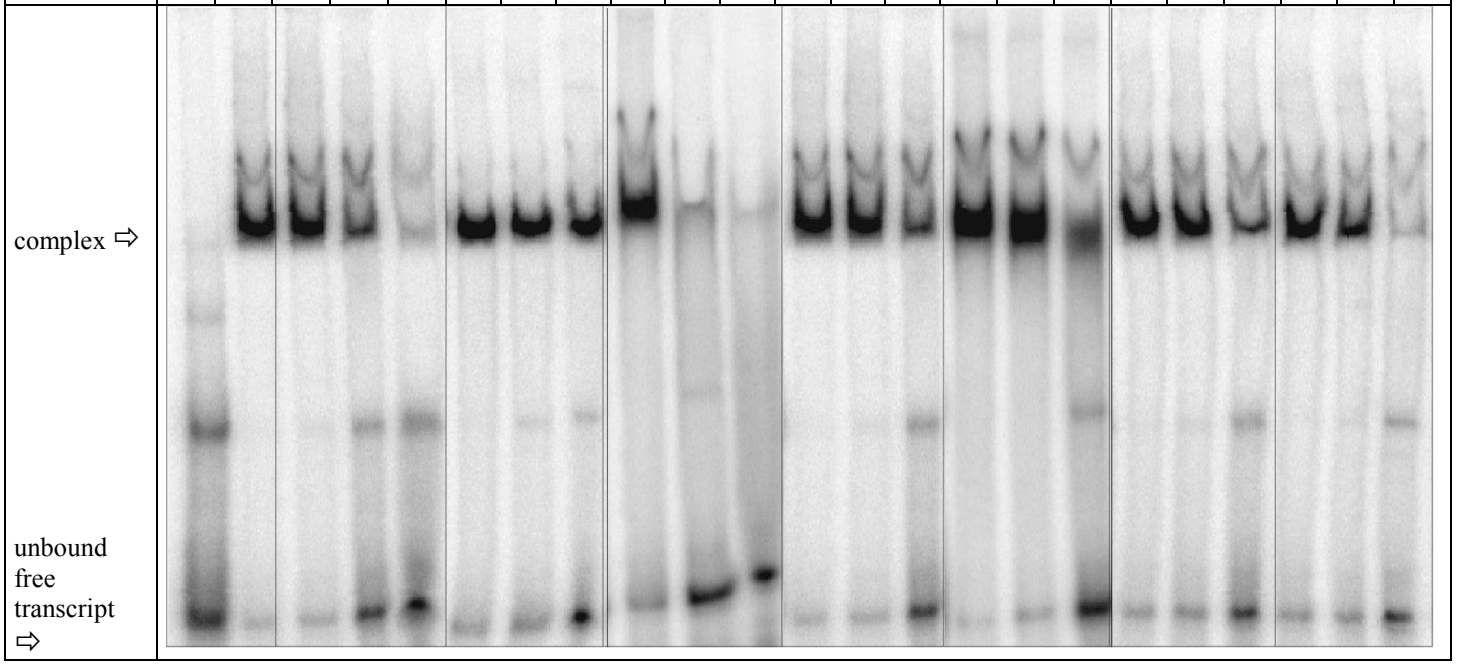


Fig. 14: Influence of various factors on RNA-binding of Mdh1p
COX2 RNA was incubated without (lane 1) and with 1 µg of purified Mdh1p-His_6 (lane 2). Lanes 3-23 acetyl-CoA, AMP, ATP, NAD^+, NADH, oxaloacetate or malate were added to the binding reactions in the final concentrations as indicated in the figure (in mM).

4 DISCUSSION

4.1 Characterisation of Idhp by mutant forms

The ability of Idh1 mutant proteins to confer normal respiratory growth was tested in the Δ*idh1* strain ΔIDH1L. While the enzymatic mutant protein Idh1p^{S92A} is not able, the RNA-binding deficient protein Idh1p$^{K182L/Y184N}$ is able to substitute the wild type Idh1p (3.1.1). To exclude the possibility that protein degradation led to the inability of Idh1p^{S92A} to substitute the wild type Idh1p, Western blot analysis of the transformants was performed. Both Idh1p^{S92A} and Idh1p$^{K182L/Y184N}$ were detectable (3.1.2). Compared to wild type Idh1p the steady state concentration of the mutant proteins is reduced, probably caused by degradation.

As evident from the complementation analysis (3.1.1) the decreased amount of mutant Idh1p$^{K182L/Y184N}$ is still sufficient for complementation of an *idh1* null mutation. Therefore, it seems unlikely that the reduction in the concentration can account for the inability of the enzymatic mutant protein Idh1p^{S92A} to complement strain ΔIDH1L.

On the basis of kinetic analysis of Idh1p^{S92A} and Idh2p^{S98A}, in which a conserved serine residue at the proposed active site of each subunit was mutated to alanine, Cupp and McAlister-Henn (1993) presented a model which proposed that Idh1p functions as a regulatory subunit while Idh2p functions in catalysis (Fig. 15). This model was supported by characterisation of conserved aspartate and isoleucine residues (Zhao and McAlister-Henn, 1997) within regions known to participate in cofactor binding in other NAD^+-specific dehydrogenases (Imada *et al.*, 1991). From these results it was concluded that Idh1p contains residues necessary for cooperative binding of isocitrate and AMP and thus appears to be the primary contributor to regulatory function. Idh2p, in contrast, appears to contribute primarily to catalytic binding of isocitrate and NAD^+. Consistent with this catalytic function, alterations within the putative isocitrate binding site of Idh2p have much more dramatic effects on catalysis than do analogous substitutions in Idh1p (Lin *et al.*, 2001). Amino acid residue replacements in the putative isocitrate binding site of Idh1p have much more moderate effects on catalysis, but many of them significantly reduce the apparent affinity for isocitrate (Lin *et al.*, 2001). This result points to a binding of isocitrate by the Idh1p site without catalytical alteration of isocitrate. All these data would explain why a disturbed regulation by Idh1p prevents the catalytic activi-ty of Idh2p and by that the energy production for a functional respiratory chain.

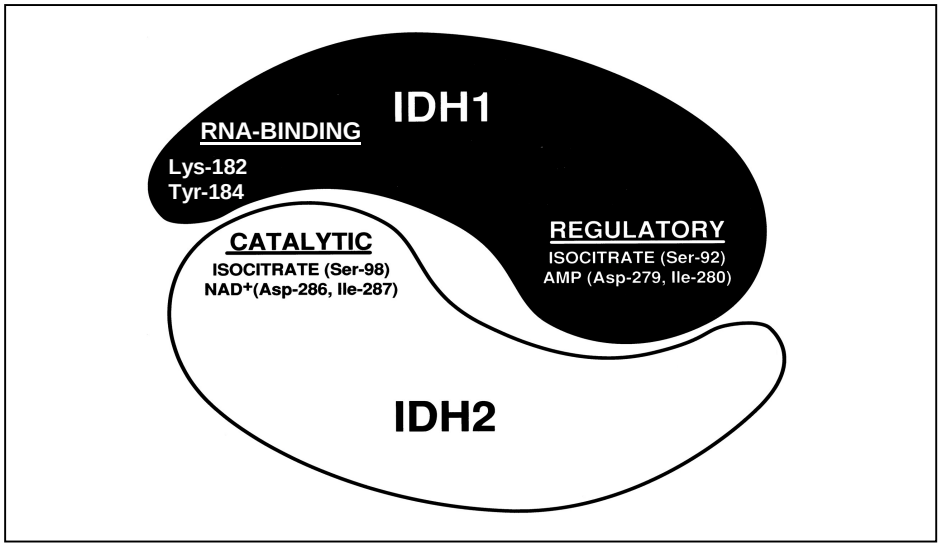


Fig. 15: Schematic presentation of important amino acids of Idhp
Studies of mutant enzymes suggest the involvement of defined amino acid residues in the enzymatic and the RNA-binding function of Idhp. The possible roles of the different subunits are indicated, (Zhao and McAlister-Henn, 1997; modified).

Another possibility besides a regulatory effect of Idh1p on the catalytic function of Idh2p would be that alterations in the active site of Idh1p could disrupt the interactions between both subunits. As both subunits are necessary for enzymatic activity (Cupp and McAlister-Henn, 1992) functionality is only guaranteed by proper interactions and therefore missing interactions could lead to the respiratory deficient phenotype. Most of the structural subunit interactions in the holoenzyme involve heterologous subunit interactions. This is suggested by the monomeric state of either Idh1p or Idh2p isolated from yeast mutants lacking the other subunit (Cupp and McAlister-Henn, 1992). Results from a yeast two-hybrid system indicate strong interaction between Idh1p and Idh2p, but no detectable interactions between identical subunits (Panisko and McAlister-Henn, 2001). In line with this observation Cupp and McAlister-Henn (1993) showed that mutant proteins assemble into heteromeric octameric complexes similar to wild type.

The result that the Idh1p^{S92A} active site mutant expressed in single- and in multi-copy is not able to complement an *idh1* null mutation (data not shown) suggests that the serine residue at position 92 in the isocitrate binding site of Idh1p is very critical for its function. The effect of the S92A mutation is remarkable, because transformants expressing other Idh1p active site mutant enzymes grow well on glycerol medium (Lin *et al.*, 2001), thus indicating no significant effect on growth due to altered kinetic properties associated with the specific residue replacements.

The observation that the RNA-binding deficient mutant protein Idh1p$^{K182L/Y184N}$ is able to confer growth on glycerol could be explained it reduced RNA-binding efficiency is only effective *in vitro* and not *in vivo*. Alternatively, the reduced RNA-binding efficiency may be sufficient to form an active respiratory chain.

To analyse the *in vivo* situation in mitochondria, protein labelling experiments of transformants expressing either the enzymatic mutant protein Idh1p^{S92A} or the RNA-binding deficient protein Idh1p$^{K182L/Y184N}$ were performed (3.1.3). Whereas *in organello* labelling did not reveal any differences between those two mutants, the steady-state level of Cox3p proved to be different. Thus the RNA-binding mutant shows the same Cox3p level like the wild type consistent with the findings of the complementation experiment that RNA-binding deficiency of Idh1p obviously does not play a major role in regulation of mitochondrial function. But the enzymatic activity of Idh1p, perhaps in combination with its regulatory function, seems to be important. Thus the enzymatic mutant shows reduced Cox3p levels similar to that obtained from an *idh1* null mutant strain. In both cases Cox3p seems to be less stable than in wild type cells. Data of synthesis and assembly of mitochondrially encoded proteins could provide an explanation for that: in the absence of Idh1p both synthesis and degradation of Cox3p is enhanced (de Jong *et al.*, 2000). Furthermore only a small fraction of the newly synthesised Cox3p is assembled into cytochrome *c* oxidase whereas unassembled protein is rapidly degraded (de Jong *et al.*, 2000). The reduced amounts of Cox3p may point to an increased degradation, while the instability of Cox3p could explain this degradation by unassembling. To a further characterisation of the enzymatic mutant steady-state levels of other proteins like Cox1p and Cox2p, which are dramatically decreased in an *idh1* null mutant strain should be defined.

If an enzymatic mutant behaves like an *idh1* null mutant strain, the questions arise: Why does the mutant show this behaviour? Is the enzymatic function of Idhp regulated ? The enzymatic activity of Idhp can be inhibited by the addition of target mRNAs (Anderson *et al.*, 2000; Elzinga *et al.*, 2000). This inhibition is relieved by the presence of the allosteric activator AMP (Anderson *et al.*, 2000), indicating that the mitochondrial AMP level can determine the switch between enzymatic and RNA-binding function. On one hand if the Idh1p is not able to switch to the RNA-binding function due to a mutation in the active site, the protein cannot act as translational repressor (de Jong *et al.*, 2000). Thus the synthesis of mitochondrial products would be disturbed and their subsequent assembly into functional respiratory complexes prevented. On the other hand if a mutation of the Idh1p prevents an interaction with Idh2p the holoenzyme also loses its RNA-binding facility. The observation that neither (di-) nucleotide

cofactors nor the substrate, isocitrate, regulate RNA-binding (Elzinga *et al.*, 1993) suggests that regions distinct from the dinucleotide binding site and from the active site are involved in binding RNA. May be an interdependence of both subunits during enzymatic and/or RNA-binding activity is required.

It cannot be ruled out that the regulation of enzymatic and/or RNA-binding activity is due to modification of the enzyme, as is shown for $NADP^+$-Idhp from *E. coli*. In this case the enzymatic activity is regulated by preventing the binding of isocitrate by phosphorylation of the active site (Dean *et al.*, 1989; Hurley *et al.*, 1990). It was mentioned by these authors that yeast mitochondrial NAD^+-Idhp is not subjected to any post-translational modification (Fox, 1996). However, close examination of a two dimensional SDS-PAGE using iso-electric focussing in the same paper reveales a minor spot for Idh1p, which could be an indication of a post-translational modification of this subunit.

It is still not known whether Idhp binds to RNA as part of the metabolon or as its own. The gel retardation assays indicate that isolated Idhp - at least *in vitro* - is able to bind RNA independent of other Krebs cycle enzymes. Although there exists an interdependence of the Krebs cycle enzymes within the intact mitochondrion (Beeckmans and Kanarek, 1987) this interdependence looks different compared to the picture drawn in the following paragraph. Thus, if the function of one part of the cycle is blocked the function of the entire cycle appears to be blocked (Kispal *et al.*, 1989; Sümegi *et al.*, 1992a, 1992b).

Gadde and McCammon (1997) made the interesting observation that yeast strains with *idh2* nonsense or null mutants grow very poorly on glycerol. These mutants have a strong tendency to accumulate extragenic mutations, termed glycerol suppressors, that enhance growth on this non-fermentable carbon source. Missense, nonsense and null mutations at the *CIT1* locus encoding mitochondrial citrate synthase, were the most common suppressors identified. In addition to Cit1p, mutations in 3 other Krebs cycle enzymes are capable of suppressing *idh2* mutations. Genetic evidence shows that glycerol suppressor mutations only occur in *idh* deficient mutants, indicating that Idhp plays a unique role in *S. cerevisiae* growing on glycerol. Mutations in other Krebs cycle enzymes do not give rise to suppressor mutations (Przybyla-Zawislak *et al.*, 1999) which indicates that a defective Krebs cycle does not lead to these mutations. It is more likely that instead of a defective enzymatic function a defective RNA-binding function of Idhp gives rise to suppressor mutations which might compensate for the absence of the Idhp RNA-binding function. Indeed, results presented in this work indicate that Cit1p and Mdh1p are able to bind mitochondrial mRNAs.

4.2 Localisation of Idhp

The application of the 2D Blue native gel-electrophoresis has shown that Idhp is localised at a position corresponding roughly to a molecular mass of approximately 300 kDa (3.2). It is likely that this complex consists of a multimer of Idhp, since the molecular weight of the usual octameric form is 320 kDa and results from yeast two-hybrid assays and affinity chromatography indicate significant physical interaction between Idh1p and Idh2p subunits (Cupp and McAlister-Henn, 1993; Panisko and McAlister-Henn, 2001). But it cannot be excluded that this complex consists of Idhp which is associated with (an)other protein(s). On the one hand the Krebs cycle in which Idhp is a key regulatory enzyme seems to form a large complex (Robinson and Srere, 1985) and for some of the Krebs cycle enzymes like Cit1p and Mdh1p an interaction has been shown (Velot *et al.*, 1999). On the other hand many of the mRNA specific translational activators display interaction with the mitochondrial ribosome (Fox, 1996) and are located at or near the inner mitochondrial membrane. Idhp as a mitochondrial RNA-binding protein could also exhibit interaction with the mitoribosome or inner membrane proteins. This assumption can be supported by the fact that the Krebs cycle is also localized at the inner mitochondrial membrane (Robinson and Srere, 1985), the site of the respiratory chain which is functionally connected with the Krebs cycle. Therefore association of Idhp with proteins of the Krebs cycle, of the mitochondrial respiratory chain complexes and/or of the mitoribosome is possible. The analysis of proteins in the complex could be performed by using antisera or by MaldiTOF. If a potential interacting protein is identified, this interaction can be verified by co-immunoprecipiation or two-hybrid assay.

Recently a membrane-associated complex containing five intermembrane space-facing dehydrogenases, the matrix-facing NADH dehydrogenase, a part of the Krebs cycle including Idh2p and an additional NADH-producing enzyme has been identified (Grandier-Vazeille *et al.*, 2001). Since theses authors also obtained information about potential interactions between this complex and other mitochondrial complexes, namely the bc_1-complex, the colorless native polyacryamide gel-electrophoresis in combination with Edman degradation and mass spectrometry seems to be a suitable tool for the identification of physiological interactions between proteins. The procedure could also used to identify Idhp as a component associated to membrane complexes.

4.3 RNA-binding properties of Cit1p and Mdh1p

In an attempt to look for RNA-binding proteins in the absence of Idhp an electrophoretic mobility shift with lysate from an *idh2* null mutant strain was performed. Surprisingly, as demonstrated in this work, other RNA-binding proteins beside the potential candidates like the known translational activators are present in yeast mitochondria.

The purification scheme permits at first an enrichment of enzymes because of the usage of Blue Sepharose. This material contains the dye, Cibacron Blue F3G-A, which has affinity for a wide variety of enzmyes. These include enzymes requiring adenyl-containing cofactors (including NAD^+ and $NADP^+$). In the next step an enrichment of nucleotide binding proteins is achieved due to Heparin Sepharose. Heparin shows a high content of anionic sulphate groups, which mimic the similarly polyanionic structure of nucleic acids. The expression of the mRNA specific translational activators of cytochrome b RNA as GST-fusion and histidine-tagged proteins pointed to a very low abundance in heterologous (*E. coli*) and in homologous (*S. cerevisiae*) systems (data not shown). Therefore it was unlikely to identify the translational activators among the RNA-binding proteins. Abundant proteins of the RNA-binding fractions were chosen, subjected to N-terminal sequencing (data not shown) and identified Cit1p and Mdh1p as RNA-binding candidates. To test whether these proteins were responsible for the observed RNA-protein complexes, lysates of both an Δ*idh2*Δ*cit1* double null mutant strain and an Δ*idh2*Δ*mdh1* double null mutant strain were applied for mobility shift assays. In both cases, strongly reduced RNA-binding activities were detectable. Further evidence for Cit1p and Mdh1p being the RNA-binding proteins was provided by analysis of enzymatic acitivities. The RNA-binding and enzymatic activities clearly coincide. However, the presence of (an) additional RNA-binding protein(s) cannot be excluded.

In mobility shift assays, competitor RNA is usually used to eliminate non-specific RNA-protein interactions. The anionic polymer Heparin can also work as a non-specific competitor. The different competitors do not work equally well for all proteins and it is recommended to determine which of the competitors allows optimal binding of the protein. Sensitivity of Cit1p and Mdh1p RNA-binding to Heparin was unexpected. However, it reminds to the Heparin inhibition of RNA-binding of Idhp (Siep, 2001). At a concentration of 600 nM, which is similar to the concentration which inhibits Idhp RNA-binding, complex formation was nearly absent. In the previous studies this Heparin sensitivity was discussed as an indication for the involvement of electrostatic interactions between the RNA and positively charged protein domains (arginine clusters). This might be the case in our study, too. Band-shift assays of this work are routinely performed in the presence of 50 mM KCl. High salt concentrations

(200 mM) only slightly decrease complex formation of the RNA-binding components. This may indicate that the Cit1p-RNA and the Mdh1p-RNA interaction is not exclusively an electrostatic interaction and may be comparable to the features of the Idhp RNA-binding.

Cit1p and Mdh1p bind to the same mitochondrial leader sequences of *COX2* and *COB* (data not shown) like Idhp which might be an indication for a general RNA-binding affinity to leader RNAs as shown for Idhp. Nevertheless these proteins are probably highly selective in their ability to bind RNA since a concentration of 2 µg competitor *E. coli* RNA representing an excess of RNA molecules with different sizes and shapes to the 3-5 ng of labelled RNA did not compete.

Thus, the conditions which were successfully used for the analysis of the specific interaction between Idhp and several 5'-untranslated regions of mitochondrial mRNAs were applicable to other factors that interact with such sequences. Not abundant protein(s) binding to the defined *COX2* RNA would not have been identified. In such a case the RNA-binding protein(s) could be purified and characterised by affinity methods yielding sufficient protein for amino acid sequencing. This allows either the protein to be directly identified by its sequence, or the isolation of cDNA clones leading ultimately to the expression as recombinant protein. Three different methods currently in use for affinity purification of RNA-binding proteins, one dating back over 20 years, and two relatively modern technologies (Smith, 1998):

(1) Direct covalent coupling of the RNA affinity substrate to CNBr-activated Sepharose

(2) Poly(A) tailing to bind the RNA affinity substrate to poly(U)-Sepharose

(3) Direct or indirect incorporation of biotinylated nucleotides to allow binding of the RNA affinity substrate to streptavidin which is itself covalently linked to a solid matrix.

Except in the case of the poly(A) tailing method, the RNA element used as the affinity matrix should generally be larger than the minimal length required for binding the protein in question, to avoid the risk that the binding site may be masked by the coupling to the solid support.

Based on the ability of RNA-binding proteins to interact in a stable fashion with RNA substrates while immobilised on solid support matrices such proteins can be detected and cloned. Detection of immobilised proteins with labelled RNA molecules is often referred to as "Northwestern" screening. Successful application of these methodologies can lead to the rapid identification and cloning of RNA-binding proteins like the RNA-binding protein G-rich sequence factor 1 (Qian and Wilusz, 1994) without the need for labour-intensive biochemical

purification and conventional cloning approaches. These methods, therefore, may be especially useful for characterising RNA-binding proteins of low abundance.

4.4 Effects of Cit1p and Mdh1p on mitochondrial translation

While the mRNA-specific factors activate the translation of one leader RNA, Idhp inhibits translation by binding to all leaders of mitochondrial RNAs. In cells lacking either one or both Idhp subunits, the Krebs cycle is blocked and synthesis of mitochondrial encoded products is activated because of the absence of translational repressor activity of Idhp (de Jong *et al.*, 2000). To study the potential role of the newly identified RNA-binding proteins Cit1p and Mdh1p in regulating translation *in organello* labelling of mitochondrial proteins in cells lacking either Cit1p or Mdh1p was performed (3.4). Both null mutants show wild type like synthesis and turnover indicating that Cit1p and Mdh1p are not acting as translational activators or repressors of mitochondrial mRNAs. Since the steady-state level of Cox3p is also comparable to that of the wild type strain, stability and/or assembly of mitochondrial translation products seems not to be disturbed. Taken together these data suggest that Cit1p and Mdh1p are not essential components of the mitochondrial translation machinery.

So the question arises: What could be the meaning of Cit1p and Mdh1p RNA-binding? If they are not involved in the stabilization of mitochondrial mRNAs, which is supported by equal amounts of the respective RNAs in Northern blot analysis (data not shown), they could be involved in directing of mitochondrial mRNAs to locations where these RNAs are translated. Since in mammals the mitochondrial malate dehydrogenase binds to protein components of the mitochondrial inner membrane, namely to complex I (Sümegi and Srere, 1984) and citrate synthase binds to the pyruvate dehydrogenase complex (Sümegi and Alkonyi, 1983) which is also interacting with complex I (Sümegi and Srere, 1980), both proteins may be located near the mitochondrial inner membrane in yeast, too. Interaction experiments have shown that pig heart mitochondrial Mdhp and yeast Citp bind to the yeast inner membrane. Binding is also observed as when both proteins are present at the same time. Therefore it can be supposed that in the yeast inner membrane there are at least two distinct binding sites, one specific for mitochondrial Mdhp, and the other for Citp (Brent and Srere, 1987). This binding is not restricted to yeast mitochondria. It was also observed in the inner membrane of a wide variety of mammalian species (Moore *et al.*, 1984). Binding of matrix enzymes, especially of citrate synthase, to the mitochondrial inner membrane seems to be widespread providing evidence for its physiological relevance. If mitochondrial Citp and Mdhp from yeast are located at the

mitochondrial inner membrane, then binding of both protein to mitochondrial mRNAs could guarantee a vicinity of these RNAs to their specific translational activators.

While increasing amounts of *COX2* leader RNA clearly inhibit the Idhp enzyme activity, this was not found for Cit1p and Mdh1p enzyme activity (data not shown). The results do not point to an interdependence of enzymatic activity and RNA-binding property of these two enzymes and do not suggest a reciprocal regulation between metabolic activity and RNA-binding. But does it mean that the RNA-binding is not relevant *in vivo* and is perhaps an evolutionary relict? The finding that Idhp binds *in vitro* much more sensibly to mitochondrial mRNAs than concomitant Cit1p and Mdh1p (data not shown) could support this idea. Since there are at least 25000 copies of Idhp per cell (Dekker *et al.*, 1991) and the steady-state concentration of mitochondrial mRNAs in derepressed growth conditions, is approximately 100 to 500 copies per transcript per cell (Müller and Getz, 1986), Idhp has the capacity to complex all mRNA molecules present. In mammals it could not be shown that Idhp binds to complex I and is located near the mitochondrial inner membrane, because of its instability in the course of preparation (Sümegi and Srere, 1984). Recently, however, an interaction of Idh2p with Cit1p and Mdh1p was reported (Grandier-Vazeille *et al.*, 2001). This opens the possibility that Idhp reveals the same location as Cit1p and Mdh1p. Therefore directing of mitochondrial mRNAs to the translational machinery could be achieved by Idhp, too. In view of a complex formed by the Krebs cycle enzymes it can be speculated that this complex allows regulatory interactions that provide a more favourable condition for enzymatic activity (Robinson *et al.*, 1987). Although the *in vitro* RNA-binding of Idhp does not require Cit1p or Mdh1p it could be possible that this complex provides *in vivo* conditions which allow or support the binding of specific RNA species. Thus, a highly integrated interplay among different metabolic pathways can be formed.

4.5 Localisation of Cit1p and Mdh1p

The exact structure of the Krebs cycle complex has still to be resolved; however there are compelling *in vitro* evidences that, in mammalian mitochondria, a channelling process occurs between consecutive enzymes of the Krebs cycle such as fumarase and malate dehydrogenase (Beeckmans *et al.*, 1989) or malate dehydrogenase and citrate synthase (Robinson *et al.*, 1987; Lindbladh *et al.*, 1994). This channelling is related to probable physical contacts between the enzymes (Morgunov and Srere, 1998). Using a 5-fluortryptophan-labelled Cit1p, Haggie and Brindle (1999) have shown by ^{19}F NMR that citrate synthase is motionally restricted in yeast mitochondria, consistent with its participation in a multienzyme complex.

In vivo demonstrations of channelling between citrate synthase and malate dehydrogenase (Lindbladh *et al.*, 1994), citrate synthase and aconitase (Velot and Srere, 2000) provided further evidence that Cit1p interacts *in vivo* with Mdh1p and Aco1p. Probably Cit1p plays a key structural role in cycle function which is independent of its catalytic function, since an introduction in *cit1* null mutant cells of a catalytically inactive but structurally unchanged Cit1p resulted in restoration of Krebs cycle function and growth on acetate (Kispal *et al.*, 1989).

In view of the metabolic connection and their location it is possible that the Krebs cycle and the mitochondrial respiratory chain form a supracomplex. First the Krebs cycle gene-rates NADH which is the source for the respiratory chain to generate energy by producing ATP causing a functional and maybe physical linkage. Second all of the enzymes, previously thought to exist in a soluble form in the mitochondrial matrix, bind to the inner surface of the mitochondrial inner membrane, where the respiratory chain is located, while purified isozymes from other cellular compartments do not possess such binding abilities (reviewed by Srere *et al.*, 1987).

To check whether the yeast mitochondrial Krebs cycle enzymes form a complex or are components of other mitochondrial complexes 2D Blue native gel-electrophoresis was performed. The functionally active HA-tagged forms of Cit1p and Mdh1p which are able to confer growth on acetate were located in a complex smaller than 250 kDa. Possibly the weak interactions between the enzymes that are favoured *in vivo* by the very high protein concentrations in the mitochondrial matrix (Aragon and Sols, 1991; Minton *et al.*, 1992) are disrupted by the dilutions that occurs during extraction of mitochondria. Another reason for the disruption of protein-protein interactions could be the choice of the detergent used for solubilisation. Possibly milder detergents as laurylmaltoside in lower concentration would allow the detection of the proposed metabolon. Since five of the Krebs cycle enzymes (fumarase, malate dehydrogenase, citrate synthase, aconitase and isocitrate dehydrogenase) from *E. coli* could be isolated as a high molecular weight complex (Barnes and Weitzman, 1986), the same is conceivable for the yeast proteins. Indeed, the separation of yeast mitochondrial complexes by colorless native polyacrylamide gel-electrophoresis led to the first detection of a membrane associated complex (Grandier-Vazeille *et al.*, 2001). It contains four Krebs cycle enzymes (Cit1p, Sdh1p, Fum1p and Mdh1p) and the matrix-facing NADH dehydrogenase Ndi1p. This opens new stratagies concerning the isolation of intact complexes from the cell and concerning the identification of new components associated with membrane

complexes. Eventually these methods lead to an confirmation of the Krebs cycle metabolon concept.

4.6 Specifity of RNA-binding

RNA mobility shift and competition assays were used to demonstrate that Cit1p and Mdh1p, purified from yeast mitochondrial extracts, bind specifically to the *COX2* 5'-leader RNA (3.6). In order to examine RNA-binding properties of highly purified Cit1p and Mdh1p a purification scheme was established by C-terminal fusion of the coding regions of Cit1p and Mdh1p from *S. cerevisiae* to a series of six histidine residues. It was shown by complementation analysis and enzyme assays that the carboxyl-terminal tags did not influence the function of these enzymes. Both proteins could be purified using affinity chromatography. This approach confirmed the ability of Cit1p and Mdh1p to dimerise because two bands, probably the histidine-tagged and the wild type form, were detected. This dimerisation in mitochondria was indicated by immunodetections of HA-tagged Cit1p and Mdh1p in 2D Blue native gel-electrophoresis, too (3.5.4). Several proteins are already known which require homomerisation to allow binding to RNA. Thus, it could be shown that the hnRNPC protein (heterogeneous nuclear ribonuclein particles) has to form homomeric complexes to get RNA-binding property. The hnRNPC protein belongs to the core proteins which bind as particle forming complexes to the pre-mRNA in vertebrates during transcription (Tan *et al.*, 2001). In *E. coli* it could be shown that BglG, an RNA-binding transcription anti-terminator, binds as dimer to the RNA (Boss *et al.*, 1999). Therefore it is conceivable that Cit1p and Mdh1p have to dimerise, too.

The histidine-tagged variants of Cit1p and Mdh1p are able to bind to *COX2* leader RNA (3.5.5). Moreover, in titration assays aimed at establishing a working protein concentration, saturation of the RNA-binding sites was achieved. Whereas binding of a protein to a single RNA site shows a single shifted band multiple shifted bands were observed. These probably correspond to the sequential filling of multiple binding sites as the protein concentration is increased. Since an intermediate sized shifted band was absent it can be assumed that the binding sites are filled almost simultaneously, indicating strong cooperativity. If multiple binding sites on the particular small RNA molecules are present the analysis will be more complicated. For a further characterisation of the supposed multiple binding sites binding studies on truncated RNA sequences have to be performed. The identification of smaller RNA-binding fragments would then allow mutational alteration and could be used to determine the identity and binding affinity of each individual binding site.

The established working protein concentration seems to represent a molar excess to the *COX2* leader mRNA because increasing amounts of the labelled RNA led to an enhanced signal of bound RNA. This fact may explain why in competition experiments using increasing amounts of cold *COX2* leader mRNA the same RNA started to compete only at a 50 fold molar excess. Calculations of the molar ratios yielded a ratio of 1 to 2 between protein (2.5 pmol) and RNA (5 pmol). This could be interpreted that 1 protein molecule binds to 1 RNA molecule which would be in contrast to the observed saturation of RNA-binding. Concerning a cooperative binding it could also be interpreted that there are 2 protein molecules binding to 1 RNA molecule and 1 of the protein molecules dissociates. Thus it could point to 2 RNA-binding sites, too. For an easier interpretation it is advisable to use competitor RNA at a level comparable to or greater than the input protein.

The antisense transcripts from the same RNA sequences are expected to have extensive secondary structures. Usually single stranded loops whose size and position are identical in sense and antisense are formed. Since Cit1p and Mdh1p had the same affinity for the sense as for the antisense RNA of the *COB* leader and the *COX2* leader/gene (3.6) both proteins seem to be secondary structure-specific in their ability to bind RNA. Gel shift analysis using *COB* leader and *COX2* leader/gene RNA as competitors implied that Cit1p and Mdh1p could exhibit a more general RNA-binding ability (3.6). This could probably be related to the length and the number of certain secondary structures which could offer multiple binding sites. The finding that the tested RNAs compete in the order of their lengths: *COX2* leader/gene RNA > *COB* leader RNA > *COX2* leader RNA may also imply a higher affinity. In summary Cit1p and Mdh1p bind specifically to all three RNA types.

Since all these RNAs have different sizes (from 104 nt for *COX2* leader until 810 nt for *COX2* leader/gene) and can presumably fold in different alternative secondary structures, additional studies are needed to elucidate the sequence/structure requirements for Cit1p/Mdh1p binding. In an attempt to identify common secondary structures, RNA structures were generated for 30°C by using the programm mfold 3.0 (http://mfold2.wustl.edu/~mfold/rna/form1-2.3cgi) with default parameters (Mathews *et al.*, 1999; Zuker *et al.*, 1999). One predicted stem loop structure which contains a “four” loop and a stem of 5 base pairs could be identified both in the *COX2* leader RNA and in the *COB* leader RNA. Similar structures with longer stems were predicted for *COB* leader RNA and *COX2* leader/gene RNA. In addition *COB* leader RNA and *COX2* leader/gene RNA show a “five” loop and a stem of 6 base pairs. Possibly these secondary structures were recognised by Cit1p and Mdh1p, but it cannot be excluded that other, may be relatively short sequences are involved in that recognition.

The mRNA sequence of the first six codons specifying the Cox2p leader peptide plays an important role in positively controlling translation (Bonnefoy *et al.*, 2001). Data about binding of the Cit1p and Mdh1p to this sequence could help to develop a model of the RNA-binding function.

First of all the RNA regions recognised by Cit1p and Mdh1p should identified. Chemical probing as a convenient and rapid approach could be used to identify nucleotides that are involved in protein binding. The most common strategies employed in chemical probing are footprinting and modification-interference analysis. In footprinting experiments, nucleotides that are protected from chemical attack in the presence of a bound protein are identified. In modification-interference experiments, nucleotides whose modification prevent protein binding are identified. Interpretation of RNA footprinting experiments is inherently ambiguous. A diminished reactivity of a nucleotide in response to binding of a protein to RNA can be due either to direct interaction of the protein with the protected nucleotide, or to a protein-induced conformational change in the RNA. Happily, both interpretations are interesting; however, it can often be difficult to distinguish between the possibilities.

To detect and analyse RNA-protein interactions *in vivo* the three-hybrid system provides a rapid and potentially versatile method. In the three-hybrid system, a hybrid RNA functions as the bridge between two hybrid proteins. Hybrid protein 1 contains RNA-binding domain 1 which is fused to a DNA-binding domain. Hybrid protein 2 contains a different RNA-binding domain, 2, which is fused to a transcription activation domain. The hybrid RNA contains recognition sites for the two RNA-binding domains. The interaction of this RNA with the two hybrid proteins is required for transcription of the reporter gene. While this assay possesses many features of the two-hybrid system for the analysis of protein-protein interactions, hybrid RNAs differ from hybrid proteins in significant aspects. The structures formed by these RNAs may often not be physiological, since the same RNA sequence placed into different contexts can adopt different conformations. However, local, relatively stable structures, such as those recognised by the iron regulatory protein 1 (Irp1p) form even in unnatural contexts so as to be recognised by their cognate protein, and can coexist in a single RNA molecule *in vivo* (Sengupta *et al.*, 1996). The stem-loop structures of these RNA elements are relatively stable. "Unstructured" RNAs might rather be able to form unproductive, alternative conformations, and could in principle limit the range of RNA-protein interactions that can be assayed. However, the formation of correct structures in only a fraction of the RNA molecules likely is sufficient to lead to transcriptional activation.

Despite the proven success of the method, it does not directly provide information about the physiological relevance of the interactions, since nuclear localisation of the interacting partners is required to obtain reporter gene expression. Moreover, many proteins (e.g. proteins with targeting sequences for other organelles) may not be amenable to this localisation, or the nuclear context may not be appropriate to detect some interactions that physiologically occur in another organelle. Indeed, alternative systems have been developed for analysis of interactions in cellular compartments other than the nucleus, but again, most of the interactions are detected out of their physiological context.

An alternative system the fluorescence resonance energy transfer (FRET) has been used for analysing ribosome function (Hardesty *et al.*, 1992). In the course of translation, for instance, changes in distance between two tRNAs or a tRNA and a ribosomal protein in the assembled tRNA-ribosome complex were observed (Odom *et al.*, 1990). Likewise, the topology of ribosome-bound messenger RNA with respect to the 3' end of 16S ribosomal RNA was evaluated by labelling the 5' or 3' end of various mRNA fragments with one and the 3' end of 16S rRNA with the other fluorophore of a FRET pair (Bakin *et al.*, 1991; Czworkowski *et al.*, 1991). In principle, the donor-acceptor distances obtained can be used as constraints in molecular modelling of the underlying RNA architecture in the absence of a high-resolution NMR or crystal structure (Tuschl *et al.*, 1994; Malhotra *et al.*, 1994). The versatility of the fluorescence labelling and analysis techniques very likely will lead to an increasing popularity of FRET in the "RNA World".

4.7 Influence of cofactors and substrates on RNA-binding

The combination of a great variety of irregular structures in RNA and the ease with which some of these can be deformed suggests that RNA-binding proteins will use a greater range of binding strategies than DNA-binding proteins and that "indirect readout" will be widespread. Therefore the number and kind of RNA-binding domains may also be widespread.

Many regulatory proteins which bind to nucleic acids contain conserved structural domains such as the RNP motif, the RGG box, or the KH motif (Mattaj, 1993; Burd and Dreyfuss, 1994). But the amino acid sequence of Cit1p and Mdh1p indicate that the RNA-binding domain of Cit1p and Mdh1p is distinct from the well characterised RNA-binding motifs. Since both proteins are involved in a biochemical pathway they are possibly in line with several other enzymes which also bind RNA and do not contain recognisable RNA-recognition motifs. Some representatives of these bifunctional proteins are dehydrogenases containing a Rossmann fold like glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and

glutamate dehydrogenase (GDH); some are (di)nucleotide binding proteins like members of the mammalian heat shock protein 70 (Hsp70p) family. All these enzymes, including Cit1p and Mdh1p, do however contain a (di)nucleotide binding domain. RNA-binding of GAPDH, GDH and Hsp70p could be competed by NAD(H), NADP(H) or ATP (Nagy and Rigby, 1995; Henics *et al.*, 1999) and prevented by the use of deletion mutants lacking the (di)nucleotide binding region (Henics *et al.*, 1999; Nagy *et al.*, 2000). These findings suggest that the (di)nucleotide binding domain serves as an RNA-binding domain, and indicate that the enzymatic function and RNA-binding are mutually exclusive. These observations led to the hypothesis that the RNA-binding domain present in these enzymes evolved from a (di)nucleotide binding site, or *vice versa* (Hentze, 1994).

A possible involvement of the (di)nucleotide binding domains of Cit1p and Mdh1p in RNA-binding was examined by competition experiments with the corresponding cofactors (3.7). While NAD, NADH and AMP did not inhibit, ATP competed for RNA-binding. This could be a hint at a structural motif responsible for recognition of ATP and distinct from the Rossmann fold which is involved in RNA-binding. The idea of a Rossmann fold-independent RNA-binding activity was already presented by Siep (2001) and supported by the finding that the RNA-binding capacity of Idhp was not affected by high concentrations of NAD^+ and of AMP. The demonstration that another adenine-containing ligand, acetyl coenzyme A, is also able to diminish the specific RNA-binding activity of Cit1p and Mdh1p confirms that possibly an adenine-binding motif enables the binding to RNA. To test whether one or two adenine-binding motifs are present ATP and concomitant acetyl coenzyme A should be compete. If the competing concentration is the same like needed for the competition of ATP or acetyl coenzyme A two motifs are present. If the competing concentration is lower than needed for the competition of ATP or acetyl coenzyme A one motifs is present. However, a unique adenine-binding motif was recently identified in nearly one-third of the structurally defined ATP-dependent proteins and belonging to 12 different fold families (Denessiouk and Johnson, 2000). This motif uses the same structural elements to recognise adenine, regardless of whether the ligand is a coenzyme, cofactor, substrate or an allosteric effector (Denessiouk *et al.*, 2001). Thus, in the group of CoA-dependent proteins Citp from the *Antarctic bacterium* DS2-3R, *Pyrococcus furiosus*, and *Gallus gallus* and in the group of NAD(P)(H)-dependent proteins Idhp from *E. coli* could be identified. This adenine-binding motif is present in "ancient proteins" common to all presentday organisms, suggesting that adenine-containing ligands and the common motif for binding them were exploited very early in evolution. Therefore it is conceivable that Cit1p, Idhp and Mdh1p from *S. cerevisiae* might also contain

this motif. The existence of such a motif could be examined by crystallisation of Cit1p and Mdh1p and analysis of their three-dimensional structures. Evidence for the involvement in RNA-binding could be obtained by the construction of respective deletion mutants.

Although the Rossman fold was a good candidate for a RNA-binding domain, other sequences and/or structures seem to mediate the observed RNA-binding of Mdh1p. Therefore the presence of a Rossmann fold in combination with an RNA-binding property without further studies cannot be interpreted as a strong implication of the Rossmann fold as a general RNA-binding domain as suggested by Nagy and coworkers (2000). Malate dehydrogenase from pig liver is able to bind the IFNγ 3'-UTR RNA. It remains to be analysed whether the Rossmann fold really mediates this RNA-binding.

An adenine-binding motif as an RNA-binding domain could also provide the regulatory linkage between the need for mitochondrial function and the rate of biogenesis, and would explain the combination of enzyme and RNA-binding activities in three single proteins from the same biochemical pathway. While the enzymatic function of Idhp seems to be regulated by binding of RNA (Anderson *et al.*, 2000), in the case of Cit1p and Mdh1p such a regulatory mechanism was not detected (data not shown). Therefore it is conceivable that sequence or structural elements which are not related to the enzymatic function are responsible for the RNA-binding property. In order to identify an RNA-binding region in Cit1p and Mdh1p the respective amino acid sequences from *S. cerevisiae* and *K. lactis* should be compared. This sequence comparison has already led to the identification of amino acids important for the RNA-binding of Idhp and could also show some interesting amino acid differences in conserved regions of Cit1p and Mdh1p. By means of site-directed mutagenesis mutant proteins can be engineered and used to study the role of RNA-binding by Cit1p and Mdh1p in mitochondria.

It has to be noted that ATP can chelate the Mg^{2+} ions in the binding assays and that lack of Mg^{2+} ions could prevent protein-RNA interaction. To exclude this possibility binding studies with increasing amounts of Mg^{2+} ions should be performed.

However, the data presented here imply that in addition to the known classes of RNA-binding proteins (with either RNA recognition motifs or a Rossmann fold), there might be a novel class of RNA-binding proteins.

4.8 Role of Cit1p and Mdh1p as RNA-binding proteins

RNA-binding proteins have been implicated in numerous different cellular processes like transcription, RNA processing and splicing, RNA stability, storage and transport of RNA, and different steps in translation. Cit1p and Mdh1p interact specifically with probably well defined RNA structures or elements, but with an lower affinity than Idhp.

Following the idea that Cit1p and Mdh1p are involved in creating an environment necessary for Idhp RNA-binding they might have a role as "RNA chaperones" like suggessted for the 70 kDa heat shock protein (Hsp70p) (Henics *et al.*, 1999). Hsp70p and likely other chaperones may facilitate, through direct RNA-binding, the unwinding of complex secondary structures. This would aid in the exposure of critical *cis*-acting sequences for other protein factors to bind and, hence, allow proper and efficient assembly of polysomes. Such "nucleic acid chaperone" function had been designated to other non-chaperone proteins, such as nucleocapsid proteins of RNA viruses (Rein *et al.*, 1998), heteronuclear ribonucleoprotein A1 (Dreyfuss *et al.*, 1993; Hamilton *et al.*, 1997), and glyceraldehyde-3-phosphate dehydrogenase (Nagy and Rigby, 1995). Alternativley, Hsp70p and Hsp110p may also function in modulating the interaction of a given mRNA with regulatory proteins that can influence mRNA stability and/or translation. In support of this, Scandurro and co-workers (1997) have recently suggested that Hsp70p is a potent regulator of complex formation between the 3'-UTR of erythropoietin mRNA and its specific binding protein, erythropoietin mRNA-binding protein. Hsps and Cit1p and Mdh1p may bind specific mRNAs, mediating an "RNA chaperone" function that might be required for proper folding of the RNA to expose critical motifs to regulatory proteins. Alternatively, these chaperones may be required in the fine regulation of messenger ribonucleoprotein complex formation with other RNA-binding proteins, through the modulation of their folding state or facilitation of their microcompartmentation. It is also possible that direct protection of RNA and/or stabilisation of RNA-protein complexes by chaperones are relevant mechanisms during various stress conditions.

The physiological role of an intramitochondrial RNA-binding of Cit1p or Mdh1p might further be supported by the variations of protein levels corresponding to the growth conditions. Transcription of *CIT1* and *MDH1* is repressed by glucose, a carbon source which does not require a mitochondrial function. But during the diauxic shift transcription of both genes is induced consistent with a requirement of a functional mitochondrion. Possibly these changes do not only reflect a general correlation between the mitochondrial protein levels but also an interdependence of RNA-binding activity and expression of respiratory chain

components. So, transcription of *PET494*, encoding a translational activator of Cox3p, is also repressed by glucose (Marykwas and Fox, 1989). Furthermore Pet494p is regulated by oxygen availability: anaerobically grown cells show one-fifth the level of expression in aerobically grown cells (Marykwas and Fox, 1989). Pet494p might modulate *COX3* translation in response to these conditions. Conditions which reveal a physiological relevance of the RNA-binding by Cit1p and Mdh1p have yet to be determined.

It has been proposed that metabolic RNA-binding enzymes participate in general regulatory circuits linking a metabolic function to a regulatory mechanism, similar to the situation of the metabolic enzyme aconitase, which also functions as an iron-responsive RNA-binding regulatory element (Constable *et al.*, 1992; Hentze, 1994). However, some workers have cautioned that some of these types of enzymes may merely represent "molecular fossils" of the transition from an RNA to a protein world and that the RNA-binding properties may not have a functional significance (Kyrpides and Ouzounis, 1995). Determination of the role, if any, of RNA-binding by Cit1p and Mdh1p must await further studies.

5 SUMMARY

The work presented in this thesis aimed at shedding more light on translational control through protein-binding to mRNAs in yeast mitochondria. As in other eukaryotes, yeast mitochondria are essential cell organelles. Most of the energy required for all cellular processes is derived from reactions taking place in mitochondria and key enzymes for major metabolic routes are localised in this organelle. Impaired function of the mitochondrion underlies a wide spectrum of degenerative diseases in man. So, a better understanding of mitochondrial biogenesis in yeast is likely to provide valuable information about mitochondrial dysfunction in man and other organisms.

1. **Isocitrate dehydrogenase** (**Idhp**) acts as a repressor of mitochondrial translation. Possible interactions with other proteins involved in translation or assembly were investigated in this work. Moreover the effects of Idhp mutations were analysed.

By means of Blue native gel-electrophoresis it was shown that Idhp is in a complex of molecular weight larger than the cytochrome *c* oxidase (250 kDa). Since Idhp is able to form homomers, these data indicate that multimers of Idhp are present in that complex.

The RNA-binding deficient protein Idh1p$^{K182L/Y184N}$ is able to substitute the wild type Idh1p and to complement a Δ*idh1* strain despite the reduced level of Idh1p. The pattern of mitochondrially synthesised proteins and the level of Cox3p is not affected in respective mutants.

The enzymatic mutant protein Idh1p^{S92A} is not able to substitute the wild type Idh1p. The level of Cox3p and Idh1p is reduced in the respective mutants. The lack of Idh1p as well as the S92A mutation of Idh1p lead to a respiratory growth deficiency and result in a reduced steady-state level of mitochondrially encoded proteins.

2. Two novel RNA-binding proteins, **Citrate synthase 1** (**Cit1p)** and **Malate dehydrogenase 1** (**Mdh1p),** were identified. Both proteins do not contain known RNA-recognition motifs. These Krebs cycle enzymes were examined for possible protein-protein interactions, specifi-city of RNA-binding, influence on translation and steady-state levels of mitochondrially encoded proteins.

Under the condition of Blue native gel-electrophoresis protein-protein interactions of Cit1p and Mdh1p with other mitochondrial proteins were not detected.

Preincubation of Cit1p and Mdh1p with a high molecular excess of heterologous competitor RNA or tRNA did not prevent complex formation with *COX2* leader RNA. Therefore it can be concluded that Cit1p and Mdh1p specifically bind to *COX2* leader RNA.

RNA-binding at high salt concentrations (200 mM) may indicate that the protein-RNA interactions are not exclusively mediated by electrostatic interactions. The Heparin sensitivity of the RNA-binding by Cit1p and Mdh1p may indicate the involvement of hydrophobic interactions.

Translation and steady-state levels of mitochondrially encoded proteins are not affected in ΔCIT1 and ΔMDH1 strains indicating that Cit1p and Mdh1p play no essential role in mitochondrial protein synthesis.

In competition experiments the binding of Cit1p and Mdh1p to the labelled *COX2* leader RNA was inhibited in the order: *COX2* leader/gene > *COB* leader > *COX2* leader RNA. *COB* leader and *COX2* leader/gene antisense transcripts had the same affinity for the proteins like the sense RNAs. Therefore the secondary structure of mitochondrial mRNAs may define the target site of Cit1p and Mdh1p.

In competition experiments the binding of Cit1p and Mdh1p to the labelled *COX2* leader RNA was inhibited by the substrate acetyl-CoA and the cofactor ATP. Other substrates or cofactors did not compete at the concentration of 5 mM.

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Curriculum vitae

Name:	Claudia Dorothea Deumer, geb. Heinze
Geburtstag, -ort:	04.12.1971 in Gera
Familienstand:	verheiratet

1978 – 1988	Polytechnische Oberschule in Gera
1988 – 1990	Erweiterte Oberschule in Gera
1990	Abitur

1990 – 1991	Praktisches Jahr in der Schwanenapotheke Ronneburg
1991 – 1997	Studium der Biologie an der Friedrich-Schiller-Universität Jena
1996 – 1997	Diplomarbeit in der Abteilung für Zell- and Molekularbiologie am Hans-Knöll-Institut für Naturstoff-Forschung e.V. in Jena unter wissenschaftlicher Betreuung von Herrn Prof. Dr. H. P. Saluz
1997	Beginn der Dissertation am Institut für Genetik der Technischen Universität Dresden unter wissenschaftlicher Betreuung von Herrn Prof. Dr. G. Rödel
1999 – 2001	Fortführung der Dissertation in der Fachgruppe für Molekularbiologie an der Universität von Amsterdam unter wissenschaftlicher Betreuung von Herrn Prof. Dr. L.A. Grivell

Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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Claudia D. Deumer